REGULATION OF POLYNUCLEIC ACID ACTIVITY AND EXPRESSION

[0001] This application is a continuation-in-part of U.S. application serial no. 10/354,903, filed January 29, 2003, which claims priority to U.S. provisional application serial no. 60/352,705 filed January 29, 2002, each of which is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0002] The invention relates to methods and systems for controlling the activity of preselected polynucleic acid molecules.

BACKGROUND OF THE INVENTION

[0003] U.S. Patent 5,597,697, hereby incorporated by reference in its entirety, discloses polymerase-based methods for controlling the activity of preselected polynucleic molecules by rendering these molecules templates for the template-directed polymerization of nucleic acids.

[0004] RNA silencing is a cellular, sequence-specific RNA degradation mechanism that occurs in a broad range of eukaryotic organisms including fungi (quelling), animals (RNA-interference, RNAi) and plants (post-transcriptional gene silencing, PTGS). In these organisms, RNA silencing is triggered by double-stranded RNA (dsRNA) and requires a conserved set of gene products. Recent reviews of RNA silencing in plants include Matzke et al , Science 293, 1080-1083 (2001); Vance and Vaucheret, Science 292,, 2277-2280 (2001); Voinnet, Trends Genet., 17, 449-459 (2001) and Waterhouse et al., Nature, 411, 834-842 (2001), and in fungi or animals, Cogoni and Macino, Current Opin. Genet. Dev. 10, 638-643 (2000); Bernstein et al. Nature, 409, 363-366 (2001); Carthew, Curr. Opin. Cell Biol. 13, 244-248 (2001); Zamore, Natl. Sruct. Biol. 8, 746-750 (2001); and Nishikura, Cell 107, 415-418 (2001), each of which is hereby incorporated by reference in its entirety.

[0005] The mechanism of RNA silencing is proposed to involve processing of a "long" inducing dsRNA molecule into dsRNA fragments of 21 to 25 nucleotides. The enzyme Dicer, a member of the RNAse III family of dsRNA ribonucleases, processively cleaves an inducer dsRNA. Successive cleavage events degrade the 21-25 nucleotide

fragments into 19-21 bp duplexes (small interfering RNAs, "siRNAs") having 2-nucleotide 3' overhangs. The siRNAs are proposed to then associate with the RNA-induced silencing complex (RISC) to target and degrade mRNA molecules having complementarity to the siRNA.In at least some systems, the siRNA strands serve as primers to render a target mRNA a template for template directed polymerization of ribonucleotides and further RNAse III-type dsRNA nuclease activity to inactivate the protein coding activity of the target molecule and generate further siRNAs.

[0006] Synthetic siRNAs are capable of inducing an RNA-silencing response in human and other mammalian cells that are not substantially capable of processing dsRNA to siRNA. Short RNA hairpins (stem-loop structures) can also be used to induce RNA silencing against RNA targets having regions of complementarity to at least one strand of the stem sequence of such the hairpin. (Discussed in Piccin et al, Nucleic Acids Res. Vol. 29, No. 12. e55, 2001, and Wesley et al., Plant J 27(6): 581-590, 2001, each incorporated by reference herein.) RNA:DNA hybrid molecules are also reported to be capable of triggering the RNA-silencing mechanism, as disclosed in published U.S. patent application serial no. 09/920,342.

SUMMARY OF THE INVENTION

[0007] A feature of certain embodiments of the invention is the incorporation of a functional polymerase binding site sequence (PBS) into a nucleic acid molecule that confers a discernible characteristic (for example via its sequence specific activity) such that the incorporation of the PBS renders the nucleic acid molecule a functional template for a given RNA or DNA template- directed nucleic acid polymerase. In the presence of the polymerase and primer, catalytic extension of the strand of nucleic acids complementary to the template occurs, resulting in the modulation (decrease or increase) of the characteristic-conferring activity of the reporter-template molecule.

[0008] The invention provides methods and compositions for inhibiting the expression of, or other activities of, selected polynucleic acid molecules, for example, specific cellular and/or viral mRNA transcripts. The invention further provides methods and compositions for inhibiting the replicative ability of specific polynucleic acid

molecules within a cell, for example viral genomes such as, but not limited to, plus or minus strand viral genomic RNA molecules.

[0009] The invention also provides methods and compositions that render a cell or multi-cellular organism resistant to viral infection. The invention further provides diagnostic methods and compositions for detecting the presence of a preselected virus in a sample.

[00010] The invention further provides methods and compositions for regulating the expression of a preselected gene in a cell, e.g. a transgene, by selectively rendering mRNA molecules encoding a transcriptional regulatory protein (or transcription-regulating RNA molecule) controlling the expression of the preselected gene to be a functional template for a template directed polynucleic acid polymerase or by otherwise selectively rendering such mRNA molecules targets of an RNA silencing mechanism in response to a preselected condition.

[00011] The invention provides a method for regulating the expression of a preselected gene in a cell, which comprises the steps of: providing at least one cell or multi-cellular organism wherein the expression of the preselected gene is under the control of a preselected transcriptional regulatory protein (or transcription-regulating RNA molecule) expressed from a gene in the cell; and causing the mRNA transcript for the transcriptional regulatory protein (or the transcription-regulating RNA itself) to serve as a template for the template directed polymerization of nucleic acids to that the activity of the transcriptional regulatory protein (or transcription regulating RNA) in the cell is diminished. The transcriptional regulatory protein can be a transcriptional repressor protein or a transcriptional activator protein. At least one of the preselected gene and the gene encoding the transcriptional regulatory protein (or transcription regulating RNA) can be a transgene. The preselected gene can be of the sort that does or does not naturally occur under control of the transcriptional regulatory protein (or transcription regulating RNA), generally or with respect to specific cell types embodying the invention. Such cells can, for example, be animal cells or plants cells and the invention further provides multi-cellular organisms comprising such cells.

[00012] The invention also provides cells wherein the expression of a preselected gene is responsive to infection of the cell by at least one predetermined virus, which cells comprise: a preselected gene, the expression of the gene being under the control of a preselected transcriptional regulator selected from the group consisting of a transcriptional regulatory protein or a transcription-regulating RNA molecule; a gene expressing the preselected transcriptional regulator, and means for causing the mRNA encoding the transcriptional regulatory protein or the transcription-regulating RNA molecule to serve as a template for the template directed polymerization of nucleic acids in response to infection by the virus so that the activity of the transcriptional regulator in the cell is diminished.

[00013] The invention further provides a method for excising a preselected DNA sequence element from a cellular genome, which comprises the steps of: providing a cell comprising a series of DNA sequences that includes an excisable sequence element that is bounded on either side by specific excision sequences, a repressible promoter operably linked to a gene encoding a site specific recombinase capable of recognizing the specific excision sequences, and a gene operably encoding a repressor protein specific for the repressible promoter; and causing the mRNA encoding the repressor protein to serve as a template for the template directed polymerization of nucleic acids so that the protein coding activity of the mRNA in the cell is diminished.

[00014] The invention still further provides cells wherein a preselected DNA sequence element is excisable from the cellular genome in response to infection of the cell by at least one predetermined virus, which cells comprise: a series of DNA sequences that includes an excisable sequence element that is bounded on either side by specific excision sequences, a repressible promoter operably linked to a gene encoding a site specific recombinase capable of recognizing the specific excision sequences, and a gene operably encoding a repressor protein specific for the repressible promoter; and means for causing the mRNA encoding the repressor protein to serve as a template for the template directed polymerization of nucleic acids in response to infection by the virus acids so that the protein coding activity of the mRNA in the cell is diminished. In one variation of the invention, the excisable sequence element comprises at least one preselected expression cassette for at least one preselected gene.

[00015] The invention also provides a method for inducing the expression of a preselected gene from a cellular genome, which comprises the steps of: providing a cell comprising a series of DNA sequences that includes a promoter, such as a constitutively-active promoter, a transiently-active promoter or an inducible promoter, linked to a preselected gene, the promoter and preselected gene being separated by a blocking sequence that is in turn bounded on either side by specific excision sequences, a repressible promoter operably linked to a gene encoding a site specific recombinase capable of recognizing the specific excision sequences, and a gene operably encoding a repressor protein specific for the repressible promoter; and causing the mRNA encoding the repressor protein to serve as a template for the template directed polymerization of nucleic acids so that the protein coding activity of the mRNA in the cell is diminished

[00016] The invention further provides cells wherein the expression of a preselected gene from the cellular genome is inducible or becomes inducible in response to infection of the cell by at least one predetermined virus, comprising: a series of DNA sequences that includes a promoter, such as a constitutively-active promoter, a transiently-active promoter or an inducible promoter, linked to a preselected gene, the promoter and preselected gene being separated by a blocking sequence that is in turn bounded on either side by specific excision sequences, a repressible promoter operably linked to a gene encoding a site specific recombinase capable of recognizing the specific excision sequences, and a gene operably encoding a repressor protein specific for the repressible promoter; and means for causing the mRNA encoding the repressor protein to serve as a template for the template directed polymerization of nucleic acids in response to infection by the virus so that the protein coding activity of the mRNA in the cell is diminished. In one variation of the invention, the blocking sequence comprises at least one preselected expression cassette for at least one preselected gene.

[00017] The invention still further provides a method for regulating the expression of a preselected gene in a cell, which comprises the steps of the steps of: providing a cell wherein the expression of the preselected gene is under the control of a preselected transcriptional regulatory protein expressed from a gene in the cell; and causing RNA silencing against the mRNA transcript for the transcriptional regulatory protein so that the activity of the transcriptional regulatory protein in the cell is diminished. The

transcriptional regulatory protein can be a transcriptional repressor protein or a transcriptional activator protein.

[00018] The invention also provides a method for regulating the expression of a preselected gene in a cell, which comprises the steps of: providing a cell wherein the expression of the preselected gene is under the control of a preselected transcription-regulating RNA expressed from a gene in the cell; and causing RNA silencing against the transcription regulating RNA.

[00019] The invention further provides cells wherein the expression of a preselected gene is responsive to the presence of polynucleic molecules having at least one region of known sequence, which cells comprise: a preselected gene, the expression of the gene being under the control of a preselected transcriptional regulator selected from the group consisting of a transcriptional regulatory protein or a transcription-regulating RNA molecule; a gene expressing the preselected transcriptional regulator; and means for rendering the mRNA of the transcriptional regulatory protein or the transcription-regulating RNA as a target for RNA silencing in response to the presence of the at least one polynucleic acid molecule comprising the predetermined sequence in the cell. The means can, for example, comprise preselected sequence of the transcriptional regulator gene or of an intermediate polynucleic molecule, that renders the RNA transcript of the transcriptional regulator gene as a target for RNA silencing as a result of the presence of the at least one polynucleic acid molecule comprising the known sequence in the cell.

[00020] The invention also provides cells wherein the expression of a preselected gene is responsive to the presence of polynucleic molecules having at least one region of predetermined sequence, which cells comprise: a preselected gene, the expression of the gene under the control of a preselected transcriptional regulator selected from the group consisting of a transcriptional regulatory protein or a transcription-regulating RNA molecule; and a gene expressing the preselected transcriptional regulator, wherein the gene expressing the preselected transcriptional regulator comprises sequence means rendering the RNA transcript of the gene as a target for RNA silencing as a result of the presence of the at least one polynucleic acid molecule comprising the predetermined sequence in the cell.

[00021] The invention still further provides a method for selectively excising a preselected DNA sequence from a cellular genome, which comprises the steps of: providing a cell comprising a series of DNA sequences that includes an excisable sequence element that is bounded on either side by specific excision sequences, a repressible promoter operably linked to a gene encoding a site specific recombinase capable of recognizing the specific excision sequences, and a gene operably encoding a repressor protein specific for the repressible promoter; and causing RNA silencing against the mRNA transcript for the repressor protein so that expression of the site specific recombinase is derepressed thereby causing excision of the excisable sequence element.

[00022] The invention also provides cells wherein a preselected DNA sequence is excisable from the cellular genome in response to the presence in the cell of a polynucleic acid molecule having at least one region of predetermined sequence, which cells comprise: a series of DNA sequences that includes an excisable sequence element that is bounded on either side by specific excision sequences, a repressible promoter operably linked to a gene encoding a site specific recombinase capable of recognizing the specific excision sequences, and a gene operably encoding a repressor protein specific for the repressible promoter; and means for causing RNA silencing against the mRNA transcript for the repressor protein in response to the presence in the cell of a polynucleic acid molecule having the region of predetermined sequence so that expression of the site specific recombinase is derepressed thereby causing excision of the excisable sequence element. In one variation of the invention the excisable sequence element comprises at least one expression cassette comprising at least one preselected gene.

[00023] The invention further provides a method for bringing the expression of a preselected gene in a cellular genome under the control of a preselected promoter, which comprises the steps of: providing a cell comprising a series of DNA sequences that includes a first promoter, for example a transiently-active promoter, a constitutively-active promoter or an inducible promoter, linked to a preselected gene, the promoter and preselected gene being separated by a blocking sequence that is in turn bounded on either side by specific excision sequences, a repressible promoter operably linked to a gene encoding a site specific recombinase capable of recognizing the specific excision

sequences, and a gene operably encoding a repressor protein specific for the repressible promoter; and causing RNA silencing against the mRNA transcript for the repressor protein so that expression of the site specific recombinase is derepressed thereby causing excision of the blocking sequence thereby operably linking the first promoter and the preselected gene. In one variation, the blocking sequence comprises at least one expression cassette comprising at least one preselected gene.

[00024] A related embodiment of the invention more generally provides a method for bringing the expression of a preselected gene in a cellular genome under the control of any preselected, proximity-dependent, *cis*-acting transcription regulating DNA element ("*cis*-acting element"), such as a repressor DNA element or promoter DNA element, which comprises the steps of: providing a cell comprising a series of DNA sequences that includes a *cis*-acting element linked to a preselected gene, the *cis*-acting element and preselected gene being separated by a blocking sequence that is in turn bounded on either side by specific excision sequences, a repressible promoter operably linked to a gene encoding a site specific recombinase capable of recognizing the specific excision sequences, and a gene operably encoding a repressor protein specific for the repressible promoter; and causing RNA silencing against the mRNA transcript for the repressor protein so that expression of the site specific recombinase is derepressed thereby causing excision of the blocking sequence thereby operably linking the *cis*-acting element and the preselected gene. In a related variation, the blocking sequence comprises at least one expression cassette comprising at least one preselected gene.

[00025] The invention still further provides cells wherein the expression of a preselected gene can be brought under the control of a preselected promoter in response to the presence in the cell of a polynucleic acid molecule having at least one region of predetermined sequence, which comprise: a series of DNA sequences that includes a first promoter, for example a transiently-active promoter, a constitutively-active promoter or an inducible promoter, linked to a preselected gene, the promoter and preselected gene being separated by a blocking sequence that is in turn bounded on either side by specific excision sequences, a repressible promoter operably linked to a gene encoding a site specific recombinase capable of recognizing the specific excision sequences, and a gene operably encoding a repressor protein specific for the repressible promoter; and means

for causing RNA silencing against the mRNA transcript for the repressor protein in response to the presence in the cell of a polynucleic acid molecule having the region of predetermined sequence so that expression of the site specific recombinase is derepressed thereby causing excision of the blocking sequence thereby operably linking the first promoter and the preselected gene. In one variation, the blocking sequence comprises at least one expression cassette comprising at least one preselected gene.

[00026] A related embodiment of the invention more generally provides cells wherein the expression of a preselected gene can be brought under the control of any preselected, proximity-dependent, *cis*-acting transcription regulating DNA element ("*cis*-acting element"), such as a repressor DNA element or promoter DNA element, in response to the presence in the cell of a polynucleic acid molecule having at least one region of predetermined sequence, which comprise: a series of DNA sequences that includes a *cis*-acting element linked to a preselected gene, the *cis*-acting element and preselected gene being separated by a blocking sequence that is in turn bounded on either side by specific excision sequences, a repressible promoter operably linked to a gene encoding a site specific recombinase capable of recognizing the specific excision sequences, and a gene operably encoding a repressor protein specific for the repressible promoter; and means for causing RNA silencing against the mRNA transcript for the repressor protein in response to the presence in the cell of a polynucleic acid molecule having the region of predetermined sequence so that expression of the site specific recombinase is derepressed thereby causing excision of the blocking sequence thereby operably linking the *cis*-acting element and the preselected gene. In a related variation, the blocking sequence comprises at least one expression cassette comprising at least one preselected gene.

DETAILED DESCRIPTION OF THE INVENTION

[00027] The term "polymerase binding site" (abbreviated PBS) as used herein is defined as a sequence element in a nucleic acid molecule that renders the molecule a template for a specified nucleic acid polymerase by mediating interaction between the polymerase and the nucleic acid molecule. Accordingly, primer binding sequences, promoter sequences, and origins of replication, as known to those in the field, are examples of polymerase binding sites. As illustrated in Orr et al, The Journal of

Biological Chemistry, 267, 4177-4182 (1992), with a number of polymerases, including HIV-RT, primer extension assays can be performed utilizing heterologous PBS sequences (those not normally used by a given polymerase) as long as there is sufficient complementarity between a region of the template molecule and the primer molecule used. An example is the "combined" template-primer molecule poly(rA)-oligo(dT).sub.12-18. Such a template sequence, which is complementary to the primer in question, also constitutes a polymerase binding site as defined herein.

[00028] A feature of certain embodiments of the invention is the incorporation of a functional polymerase binding site sequence (PBS) into a nucleic acid molecule which is chosen for its ability to confer a discernible characteristic (for example via its sequence specific activity) such that the incorporation of the PBS renders the nucleic acid molecule a functional template for utilization by a predetermined RNA or DNA template-directed nucleic acid polymerase. In the presence of the polymerase, suitable primer molecules, and any necessary accessory molecules, catalytic extension of the strand of nucleic acids complementary to the template occurs, resulting in a partial or total elimination of (or increase in) the characteristic conferring activity of the reporter-template molecule described due to the effects of the complementary strand or other polymerase-mediated effects.

[00029] A reporter template polynucleic acid molecule according to the invention is a polynucleic molecule (DNA or RNA) that confers some discernable characteristic, in vitro and/or in vivo, for example a cell phenotype altering activity.

[00030] The direct incorporation of a PBS sequence or sequences into, for example, an RNA reporter strand may be achieved by the ligation of double stranded DNA restriction enzyme fragments containing the PBS sequence into appropriate restriction sites of a double stranded DNA molecule which is a template for the transcription of an RNA reporter strand by a DNA-dependent RNA polymerase. Alternatively the incorporation of the PBS sequence can be achieved through site-directed mutagenesis of such a DNA molecule, the total chemical synthesis of the novel RNA reporter molecule (by, for example, the phosphoramidite method using an Applied Biosystems Model 392 DNA/RNA Synthesizer), or by any other method known in the field.

[00031] Examples of the activity of the reporter-template RNA molecule include, but are not limited to, the following types. The RNA can be a messenger RNA (mRNA) coding for a protein that confers a discernible characteristic. In this case the preferable form of the assay is cell-based such that the reporter-template RNA is produced within a suitable host cell along with the components necessary for the extension of the nucleic acid strand complementary to the reporter-template molecule, specifically the appropriate RNA-dependent nucleic acid polymerase and its necessary primer molecule if any. The characteristic-conferring protein may be, but is not limited to, an enzyme catalyzing a color reaction such as beta-galactosidase (catalyzes the chromogenic conversion of the substrate 5-bromo-4-chloro-3-indolyl-beta-D-galactoside), an enzyme conferring antibiotic resistance, such as beta-lactamase, which confers resistance to ampicillin, or an enzyme conferring the metabolic complementation of an auxotrophic state. For cell-based and multi-cellular organism-based embodiments of the invention, the characteristic conferring activity can relate to any alteration of cellular phenotype.

[00032] Alternative positions for the incorporation of a PBS into an mRNA molecule with respect to the positions of the start codon and stop codon of the mRNA molecule include the following. The PBS can be incorporated into the 3' non-coding sequence of the mRNA. The PBS can also be incorporated into the protein coding sequence of the mRNA. Incorporation of the PBS into the coding region is appropriate as long it does not change the coding sequence such that a non-functional characteristic-conferring protein is produced. The PBS may also be incorporated within one or more introns within an mRNA transcript. Finally, the PBS can be incorporated into the 5' non-coding region of the mRNA. This region contains the ribosome binding site and other regulatory sequences. Polymerase activity initiated by binding at the PBS in any of these regions can prevent translation of the mRNA by at least one of several mechanisms such as a direct block of sense strand translation by the complementary strand, the complementary strand-facilitated degradation and/or modification of the sense strand by enzymes such as RNAse H (specifically hydrolyzes the phosphodiester bonds of RNA in RNA:DNA duplexes to generate products with 3' hydroxyl and 5' phosphate ends.) and RNAse III (degrades RNA:RNA duplexes), e.g., E. coli RNAse III, interference with the RBS by formation of its complementary strand and/or its degradation. Binding of the polymerase

to its PBS without the ensuing synthesis of the complementary strand may also prevent translation due to steric interference with the translational apparatus, in particular this may be expected to occur if the PBS sequence is located near the RBS or between the RBS and the end of the protein coding sequence, but not in the 3' non-coding region or necessarily at other sites 5' to the RBS in the 5' non-coding region. The choice of PBS incorporation sites will be partly influenced by the need or lack of need to specifically determine the differential effects agents have on polymerase/PBS binding and on complementary strand extension by the polymerase. The invention is not limited by the use of single or multiple PBS sequences incorporated into one or more of the alternate positions described in the same mRNA molecule.

[00033] The invention is not limited by the means of incorporation of a PBS sequence into a polynucleic acid molecule to render it a template. In addition to altering the sequence of polynucleic acid molecule (or gene encoding it), these means also include the generation of a heterologous PBS by providing a primer sequence sufficiently complementary to a region of the polynucleic acid molecule without any modification of the template itself. The invention is not limited by the singularity, multiplicity, or position of PBS sequences incorporated into a reporter-template or other target polynucleic acid molecule nor is the invention limited by the nature of the discernible characteristic conferred by the reporter-template molecule or any target template molecule according to the invention.

[00034] For example, the reporter-template RNA molecule is a regulatory RNA controlling the expression and/or other activity of one or more genes or gene products that confer a discernible characteristic. In this case also, the components comprising the invention are preferably produced within a suitable host cell.

[00035] In another example, the reporter-template RNA molecule is a catalytic RNA molecule that confers a discernible characteristic directly, by virtue of its catalytic activity. This activity may include, but is not limited to the catalysis of color reactions. The catalytic reporter-template RNA molecule described can also be an RNA:DNA hybrid in which the incorporated primer and perhaps some other sequence of the molecule is composed wholly or partly of deoxyribonucleic acids.

[00036] The preceding discussion and examples also illustrate the manner in which any specific RNA molecule, whether naturally-occurring or genetically engineered, including but not limited to specific mRNA molecules and viral genomic RNA molecules, can be targeted according to the invention by one or more RNA-dependent nucleic acid polymerases, i.e., RNA-dependent RNA polymerases or reverse transcriptases, to inhibit one or more activities of the targeted RNA molecule.

[00037] In reporter template embodiments related to DNA-dependent DNA Polymerases, the reporter-template molecule can, for example, be a catalytic DNA molecule that confers a discernible characteristic directly, by virtue of its catalytic activity. This embodiment is analogous to that for RNA- dependent nucleic acid polymerases in which the reporter-template is a catalytic RNA. Functional interaction of a DNA-dependent DNA polymerase and the reporter-template reduces the catalytic activity of the reporter-template and provides an assay for inhibitors and activators of polymerase activity as previously described.

[00038] In reporter template embodiments related to DNA-dependent RNA Polymerases the reporter-template may be a single stranded, partially double stranded, or double stranded DNA molecule that confers a discernible characteristic, for example, in a direct manner via its sequence specific catalytic activity. Such a reporter-template contains the promoter sequence and other sequences necessary to direct transcription of RNA. Functional interaction of a DNA-dependent RNA polymerase and the reporter-template reduces the latter's catalytic activity.

[00039] The cell-based embodiments of the invention may comprise any suitable host cell as long as the components of the assay are functional in the cell type in question. These host cell types may include but are not limited to mammalian cells, avian cells, fish cells, insect cells, plant cells, yeasts and bacteria.

[00040] RNA-degrading and/or modifying enzymes that may be used in accordance with the invention, as described, may for example be endogenous to the host cells or introduced by genetic methods to the host cells. In the case of in vitro, non-cell-based embodiments of the invention, enzymes such as nucleases can be directly provided to the composition or mixture comprising the invention.

[00041] Embodiments related to systems and methods for controlling the activity of polynucleic acid molecules within cells and multi-cellular organisms

[00042] In this embodiment, the invention is used to control gene expression and, in general, the activity of any nucleic acid strand of interest. By operably linking the activity of a nucleic acid polymerase to a nucleic acid strand of interest, the expression of, or other activity of, the nucleic acid strand can be controlled by the addition and subtraction of the polymerase itself, cofactors of the polymerase such as but not limited to primer molecules, or inhibitors and activators of the polymerase in question. In one embodiment of the invention, the cellular expression of a specified protein is controlled by operably linking the activity of an RNA-dependent nucleic acid polymerase to an mRNA molecule coding for the protein.

[00043] In another embodiment of the invention, the polynucleic acid molecule rendered subject to polymerase-mediated regulation is a viral replicative polynucleic acid molecule and the activity inhibited by the polymerase is the replicative activity of the polynucleic acid.

[00044] In still another embodiment of the invention, the polynucleic acid molecule rendered subject to polymerase-mediated regulation is a viral mRNA molecule or viral regulatory RNA molecule, and the activity inhibited by the polymerase is the viral function of the protein encoded by the viral mRNA molecule or the viral function of the viral regulatory molecule.

[00045] In a further embodiment of the invention, the polynucleic acid molecule rendered subject to polymerase-mediated regulation comprises a polynucleic acid aptamer (DNA or RNA) molecule having specific binding activity to one or more ligand molecules, as known in the art. Thus, the invention provides a method for reducing the binding between a polynucleic acid aptamer and a ligand to which the aptamer has characteristic binding affinity by rendering the aptamer a template for the template directed polymerization of nucleic acids by a template directed nucleic acid polymerase. Said polymerization can reduce the binding between the aptamer and its ligand by disrupting the characteristic ligand-binding secondary structure of the aptamer and/or by resulting in the degradation of the aptamer.

[00046] As disclosed above, in other embodiments of the invention the polynucleic acid molecule rendered subject to polymerase-mediated regulation is a regulatory polynucleic acid molecule or a catalytic polynucleic acid molecule.

[00047] According to the invention, the polymerase or polymerases selected to inhibit the expression or other activity or function of a targeted polynucleic acid can be provided to the cell(s) in several ways. First, the polymerase may be a viral polymerase that is provided to the cell as a result of infection of the cell by a virus via, for example, (i) translation of polymerase-encoding mRNA which is part of an infecting virion, (ii) translation of polymerase-encoding mRNA which is transcribed from the viral genome within the cell and/or (iii) when the viral polymerase is a component of the virion itself (e.g., as can be the case for HIV) by direct delivery to the cell as a result of infection of the cell by the virus. Second, the cell may be genetically modified to express a suitable polynucleic acid polymerase, which polymerase is not otherwise expressed by the cell at all or at least not ordinarily expressed to a sufficient level to effectuate the desired polymerase-mediated inhibition. Third, the polymerase can be a polymerase endogenous to the cell(s) but which under normal cellular conditions is not substantially directed to a preselected polynucleic acid molecule for which inhibition is desired.

[00048] <u>Inhibition of multiple target molecules</u>

[00049] Plant and animal cells can be engineered to express several different sequence specific primers targeting different transcripts and/or genomic elements for one or more specific viruses thereby imparting multiple viral resistances to the cell(s).

[00050] <u>Endogenous or exogenous polymerases</u>

[00051] A polymerase selected for use according to the invention to inhibit the activity of a target polynucleic acid molecule can, for example, be endogenous to the cell or multi-cellular organism, can be provided by a virus infecting the cell(s), or can be provided to the cell(s) or multi-cellular organism by genetically modifying the cell(s) or multi-cellular organism, according to standard methods, to express the polymerase. In some cases an identified cell type may be known to possess a desired, endogenous, template-directed nucleic acid polymerase activity although the enzyme responsible for the activity has not been identified. In this case, selecting a template directed nucleic

acid polymerase for use according to the invention can comprise constituting the elements of the invention within cells of the cell type.

[00052] Selecting RNA or DNA polymerases

[00053] For the suppression of mRNA transcripts or other RNA molecules, both RNA-directed DNA polymerases or RNA-directed RNA polymerases are suitable according to the invention. For the suppression of viral genomic replication in particular, the target suppressing polymerase selected may, for example, be of the type not utilized in normal viral replication. For example, the replication intermediates of RNA viruses that normally utilize a viral RNA-directed RNA polymerase for replication can be targeted by an RNA-directed DNA polymerase so that a replication-incompetent RNA:DNA hybrid is formed.

[00054] For example, in one embodiment of the invention, hoofed mammals susceptible to Foot and Mouth Virus (FMV) are genetically modified to make them resistance to infection by FMV. FMV is a single stranded, plus strand RNA virus. Normal replication of the FMV genome is dependent on an RNA-dependent RNA polymerase. There is no DNA replication intermediate in the FMV replication cycle. Thus, in one implementation of the invention, FMV susceptible animals are genetically modified to express a Reverse Transcriptase (RT) and primer molecule with a region of complementarity to the FMV plus strand which, together, target and inhibit replication of the plus strand RNA of FMV. In contrast to the double stranded RNA intermediate characteristic of FMV replication, FMV is not at all naturally equipped to replicate or serve as a template for transcription when the plus strand is complexed with complementary DNA to form an RNA:DNA hybrid, such as that formed by interaction with the RT.

[00055] RNAses or DNAses, integral to the polymerases, endogenous to the cell or engineered to be expressed in the cell, may also be employed according to the invention to degrade the target molecule as the selected polymerase utilizes the target as a template or at any point thereafter. Hence, in the FMV example above, if an RT having an integral RNAse H, such as HIV-RT, is used, the plus strand FMV RNA will be digested during its reverse transcription. Examples of reverse transcriptase enzymes that lack RNAse

activity and that can be used according to the invention include the various RNAse H domain/activity deficient mutants of HIV-RT that are known in the art. Members of the Dicer endonuclease family (Bernstein et al. Nature, 409, 363-366, 2001; Hutvagner et al. Science, 293, 834-838, 2001; Nicholson et al. Mamm Genome, 13(2), 67-73, 2002) of RNAse III type endonucleases are examples of nucleases suitable for digesting double stranded RNA molecules formed as a result of rendering an RNA molecule a template for an RNA-dependent RNA polymerase according to the invention.

[00056] Any suitable type of primer molecule can be used to specifically prime the desired polymerase activity. One method of targeting a reverse transcriptase such as HIV-RT or HIV-RT derived polymerase to a target preselected polynucleic acid molecule in a cell is to provide the cell with the expression of an HIV-RT primer such as human $tRNA^{Lys\text{-}3}$ modified so that its 3' polynucleotide sequence is complementary to a sequence of the target molecule. Such expression may be provided by introduction of a primer transgene into a cell, or multi-cellular organism, as part of an expression cassette, and/or by genetically modifying a preexisting cellular gene for $tRNA^{Lys\text{-}3}$, for example, by homologous recombination techniques. The polymerase binding site and primer requirements for HIV-RT are disclosed in Weiss et al, Gene, 111, 183-197 (1992) and Kohlstaedt and Steitz, Proc. Natl. Acad. Sci. USA 89, 9652-9656 (1992). Those for HBV-RT are disclosed in Wang and Seeger, Cell, 71, 663-670 (1992).

[00057] Many different RNA-dependent RNA polymerases (RdRPs) can be used according to the invention. These include, for example, RdRPs of RNA viruses and cellular RdRPs of plants. Those skilled in the art will appreciate that any sort of cell can be genetically engineered to express a selected RdRP gene. Any suitable type of primer molecule can be used to specifically prime the desired RdRP activity according to which RdRP is selected. Many RNA virus genomes and satellite RNA virus genomes have a 3' integral primer, which comprises secondary structure resembling tRNA. Examples of viruses with tRNA-like structure at the 3' end of their genome include: Tymoviruses (e.g. turnip yellow mosaic virus; Bromoviruses (e.g. brome mosaic virus); Cucumoviruses (e.g. cucumber mosaic virus), Hordeiviruses (e.g. barley stripe mosaic virus), Tobamoviruses (e.g. tobacco mosaic virus), Tobraviruses (e.g. tobacco rattle viruses). One method of targeting an RdRP such as BMV-RdRP, to a target preselected RNA

molecule in a cell is to provide the cell with the expression of a primer molecule based on the 3' integral primer of the virus and modified so that its 3' polynucleotide sequence is complementary to a sequence of the target molecule to be rendered a template.

[00058] Mispriming techniques

[00059] Cells engineered with primers that redirect a viral polymerase to an "inappropriate starting point" along the viral genome, i.e., different than the primer binding site characteristically used by the virus, may be used to prevent the formation of full length viral replication intermediates. For example, engineered primers binding downstream (in the 3' direction with respect to the primer) of the virus' normal primer binding site cause a non-full length primer extension product to be formed rather than the virally-normal, replication-competent product. As a result, polymerase mediated synthesis of the full-length "virally-normal" product is at least partially inhibited. In one embodiment of this method, the viral polymerase contains an integral or closely associated nuclease, such as an RNAse H, that degrades the template during template-directed polymerization. In this manner, synthesis of the virally-normal, full-length product is precluded since some part of the template strand downstream of the PBS normally used by the virus is degraded, thereby preventing the formation of the characteristic full –length product from the characteristic PBS. An example of a polymerase with an RNAse H domain and which uses a characteristic viral PBS is HIV Reverse Transcriptase (HIV-RT).

[00060] Control of activity of preselected cell-encoded polynucleic acid molecules by redirection of viral polymerase activity to the cell-encoded molecules

[00061] According to one embodiment of the invention, cells may be engineered to express specific primer molecules that cause preselected cell-encoded polynucleic acid molecules_ to serve as templates for viral polymerase activity, so that, upon infection of the cells with a virus providing the polymerase, the activity of the preselected polynucleic acid molecules coded by the genes (specific mRNAs molecules, regulatory RNA molecules, etc.) is modulated (reduced or increased) as a result of the functional interaction between the viral polymerase and the polynucleic acid molecule.

[00062] In another embodiment of the invention, a cellular gene (of endogenous origin or foreign origin with respect to the cell) is genetically modified so that the single stranded polynucleic acid molecule product of the gene (ssRNA or ssDNA) comprises a functional polymerase binding site sequence (for example, as a result of altering the sequence of the gene or by providing for the cellular expression of a suitable primer) which renders the polynucleic acid molecule a template for the viral polymerase in the presence of the viral polymerase in the cell, thereby reducing the activity of the polynucleic acid molecule.

[00063] Cellular resistance to virus via redirection of viral polymerase activity to viral polynucleic acids or polynucleic acids produced using the viral genome as a template

[00064] According to one embodiment of the invention, cells may be engineered to express specific primer molecules so that, in infected cells, preselected viral RNA molecules are targeted and their normal activity inhibited by at least one viral polymerase provided by the virus itself. In this context "provided" by the virus means that the polymerase polypeptide is already present in the virion when it enters the cell or that the virion contains mRNA coding for the polymerase, which mRNA is translated once in the cell or that the viral genome is capable of serving as a template for the transcription of mRNA coding for the polymerase, which mRNA is normally transcribed and translated in the cell. In this embodiment of the invention, the viral polymerase is directed to viral polynucleic acids or regions thereof, which do not normally serve as templates for the viral polymerase. In this manner, the normal activity of the viral nucleic acids is inhibited. For example, in the case of a virus providing a reverse transcriptase or an RNA-dependent RNA polymerase, a cell or multi-cellular organism can be genetically modified to express primer molecules that direct the polymerase to use an mRNA transcript of the virus as a template for the template directed polymerization of nucleic acids. In this manner, translation of the viral mRNA transcript is inhibited.

[00065] Polymerase-mediated viral infection detection systems

[00066] A biological assay for the presence or absence of particular viruses in a sample or in the environment in general is provided according to the invention by modifying cells to express cellular transcripts which have reporter activity and which are

operably –linked to the viral polymerase activity when the polymerase is provided by infection of the cell, so that the reporter activity is inhibited by the viral polymerases of the particular infecting virus(es). This viral infection detection system may, for example, be employed in a laboratory assay format utilizing test cells, i.e., a test cell line subjected to samples. These cells may be of the type or derived from the type of cells that are naturally infected by the particular viruses for which the assay is developed. Alternatively, cells which are not normally susceptible to infection by the particular virus(es) for which it is desired to develop assay cells, may be engineered to have the appropriate susceptibility to infection. This can be achieved by various methods known in the art such as, but not limited to, engineering the subject cells to express a virus receptor protein or receptor glycoside which is otherwise missing from the subject cell or expressed on at a low level by the cell. Those skilled in the art will understand that the method will be tailored to the specific virus and cell type in question.

[00067] Multi-cellular organisms can also be genetically modified to embody this type of viral detection system generally, or in one or more selected tissues or cell types. In one embodiment of the invention, a plant is engineered to embody the viral detection system. A suitable reporter gene for a plant can be, for example, a gene conferring a particular pigmentation or coloration, the absence of the expression of the gene being readily discernable. In this manner, substantially real-time monitoring of pathogen activity in a field of crops can be accomplished and appropriate steps to limit further crop damage can then taken. In another embodiment of the invention, animals such as, but not limited to, livestock can be genetically modified to similarly embody the viral detection system in one or more tissues and hence report the presence of viral infection.

[00068] In one method of the invention, agricultural crops in the same field or commonly raised animals generally embody a viral infection detection system according to this or any viral infection detection embodiment of the invention. In this manner, real-time monitoring of pathogen activity can be accomplished and appropriate steps to limit further damage, such as destruction of the affected section of crops or segregation of affected livestock, can then taken. In another method according to the invention "detection" organisms embodying a viral infection detection system according to the

invention are provided within a field of agricultural crops or among a group of commonly raised livestock that generally do not embody such a detection system.

[00069] One embodiment of the invention provides a diagnostic method, and cell compositions therefor, for determining the presence or absence of a pre-specified virus in a sample comprising the steps of: providing tests cells genetically engineered so that a preselected reporter template molecule expressed within the cells becomes a functional template of a template directed nucleic acid polymerase as a result of infection of the cells by virus present in the samples; contacting a sample potentially containing virus with the test cells; and determining whether the discernable characteristic-conferring activity of the reporter-templates of the test cells is modulated, thereby indicating presence of the virus in the sample.

[00070] A related diagnostic embodiment comprises performing the steps in parallel with the test sample and a negative control sample not containing the virus, and comparing the result obtained using the test sample to the results obtained from the negative control sample to determine whether the test sample contains the virus. Still another embodiment of the diagnostic method comprises performing the steps in parallel with the test sample and a positive control sample containing a control virus to determine whether the test sample contains the virus. For safety, when the virus for which testing is being performed is a pathogenic virus of humans, other animals or plants, the virus used as the positive control need not actually be pathogenic but merely needs to resemble the actual pathogenic virus with respect to mimicking its performance in the assay. In a still further related embodiment, the diagnostic method is performed using the test sample and both the positive and negative control samples.

[00071] The discernable characteristic-conferring activity of the reporter template may be of any detectable sort including, but not limited to, directly or indirectly repressing the expression of another gene that has reporter activity by, for example, virtue of coding for a protein having reporter or selectable marker activity as known in the art, whereby said repression is relieved as a result of the reporter template being made to serve as a template upon viral infection.

[00072] The test cells of these diagnostic embodiments may be cultured in any appropriate format, including, but not limited to, liquid suspension culture or culture on a surface submerged in growth media or on a surface of growth media. Further, the steps of the diagnostic method may, for example, be performed in one or more of any sort of appropriate tube, well plate, vessel or container as known in the art.

[00073] It should be understood that in the performance of the steps of the diagnostic method, changes in the reporter activity of the tests cells caused by virus can include the change caused by the original virus particles in the sample and also progeny virus which propagates from this original virus. Thus, according to the invention, after treating the tests cells with a sample, the mixture can optionally be incubated for varying amounts of time to allow for viral propagation to occur. In another embodiment, virus that may exist in a sample can be propagated in other cells before testing to amplify the amount of virus in the original sample. The cells used for propagation of the virus in this embodiment need not be test cells, but can be any sort of cells permissive for propagation of the virus. In a related embodiment, the propagator cells, having been incubated with the original sample, are separated from the putatively-propagated virus containing mix/culture and the cell-free mix is then introduced to the test cells.

[00074] The cell-based diagnostic assay embodiments of the invention can benefit in sensitivity by the use of test cells which are highly prone to infection by the subject virus(es) for which the diagnostic assay is designed. Accordingly one embodiment of the invention provides that the test cells are mutated and selected to be more highly prone to infection by the subject virus(es). Such mutation may, for example, be random in response to a mutagenic treatment followed by selection or may involve genetic engineering. For example, a cell being designed for use as a test cell can be genetically engineered to express a higher level of the receptor that the subject virus(es) uses to enter the cell.

[00075] <u>Transcriptional regulatory protein embodiments of the invention</u>

[00076] In this embodiment of the invention, the RNA molecule made to be a template for a viral polymerase is an mRNA coding for a transcriptional activator protein or a transcriptional repressor protein that activates or represses, respectively, the expression of

one or more other preselected genes of any sort including, but not limited to: reporter genes, lethal/toxic genes, essential genes, cell phenotype altering genes, herbicide resistance genes, and genes which themselves code for a transcriptional regulatory protein controlling the expression of still one or more other genes. Further, the invention provides that the preselected genes can be of the sort found in nature under the control of the regulatory protein or can be genes not found in nature under control of the particular regulatory protein.

[00077] Derepression of expression embodiments of the invention

[00078] In one embodiment of the invention, an RNA molecule made to be a template for a viral polymerase is an mRNA coding for a transcriptional repressor protein which represses the expression, or is engineered to repress the expression of, one or more other preselected genes such as, but not limited to, a reporter gene or a suicide gene which causes an infected cell to die, thus limiting spread of the viral infection within a multi-cellular organism or to other organisms.

[00079] For example, a cell can be genetically modified so that the expression of the luciferase gene is repressed by a repressor protein that is expressed within the cell. The repressor protein used may be endogenous to the cell or expressed within the cell as a result of genetically modifying the cell. In any case, in this embodiment, the repressor protein transcript is operably-linked to the activity of one or more selected viral polymerases in the fashion of the invention. Upon infection of the cell(s), direction of the viral polymerase to this repressor gene transcript prevents its expression, thereby leading to derepression of, in this example, the luciferase gene. Addition of the luciferase substrate and action thereon by the luciferase enzyme, if its expression has been de-repressed, will cause light to be emitted thus indicating the derepression. Another example of a reporter gene that can be used is green fluorescent protein (GFP) or any of its numerous derivatives known in the art. In this manner, a test system for the presence or absence of particular viruses can be provided.

[00080] The repressor protein coded by the repressor mRNA transcript may be an inhibitory transcription factor capable of directly interacting with the regulatory sequences of the repressed gene, whether endogenous or engineered, as known in the art

or may indirectly interact with other biomolecules present in the cell to repress the repressed gene. For example, for plant embodiments of the invention, Tn10 tet repressor systems, as described in Gatz and Quail (1988) and Gatz, et al. (1992), can be adapted for use according to the invention. In this system, a modified Cauliflower Mosaic Virus (CaMV) 35S promoter containing one or more, e.g. three, tet operons is used; the Tn10 tet repressor gene produces a repressor protein that binds to the tet operon(s) and prevents the expression of the gene to which the promoter is linked. The presence of tetracycline inhibits binding of the Tn10 tet repressor to the tet operon(s), allowing free expression of the linked gene. Gatz and Quail, "Tn10-encoded tet repressor can regulate an operator-containing plant promoter," Proc. Natl. Acad. Sci. USA, 85:1394-1397 (1988) and Gatz, et al., "Stringent repression and homogenous derepression by tetracycline of a modified CaMV 35S promoter in intact transgenic tobacco plants," The Plant Journal, 2:397-404 (1992), hereby incorporated by reference in their entireties. However, the present invention is not concerned with regulation of the system by tetracycline, although such regulation for which the prior system was designed may optionally be left intact according to the present invention. Instead, according to the invention, the transcript for the repressor protein is made to serve as a template for the viral polymerase, thereby reducing the production of the repressor protein and activating expression driven by the modified CaMV 35S promoter.

[00081] Tetracycline responsive promoter systems known in the art for mammalian cells can similarly be adapted for use according to the invention. For example, US Patents 5,723,765 and 6,242,667 disclose suitable repressor systems, and are hereby incorporated by reference in their entireties. Those skilled in the art will appreciate that there are many characterized natural repressor and genetically engineered transcriptional activator protein and transcriptional repressor protein systems for plant and animal cells that can be routinely adapted to function according to the invention as described herein. Those skilled in the art will further understand that any gene of interest may be placed under the control of any operable transcriptional repressor or activator by genetically modifying a cell (stably or transiently) and that the repression or activation can be selectively modulated, according to the invention, by rendering the mRNA transcripts

coding for the transcriptional regulator protein as functional templates for a template directed polynucleic acid polymerase.

[00082] One related embodiment provides introducing a series of sequences into a cell or multi-cellular organism that includes an excisable sequence element that is in turn bounded on either side by specific excision sequences, a repressible promoter operably linked to a gene encoding a site specific recombinase capable of recognizing the specific excision sequences, and a gene operably encoding a repressor specific for the repressible promoter. According to the invention, rendering the transcripts encoding the repressor protein as a template for template directed polymerization of nucleic acids, e.g. in response to some condition, reduces the expression of the repressor protein, thereby derepressing expression of the recombinase, thereby causing excision of the excisable sequence, thus altering the sequence of the DNA molecule in which the excisable sequence was previously found. In this manner, polymerase mediated inhibition of the protein coding activity of a preselected mRNA can be used to induce a genotypic change in a cell. The presence or absence of the genotypic change in the cell or within a population of such cells can be detected by standard methods, for example, PCR amplification. The resulting genotypic changes present in the cell and its progeny, if any, serve as a record of exposure to the condition that caused the genotypic change to occur, for example, infection of the cell by a particular virus. Thus, a variation of this embodiment of the invention provides another sort of diagnostic assay for viral infection.

[00083] Another related embodiment provides introducing a series of sequences into a cell or multi-cellular organism that includes a promoter, such as a constitutively-active promoter, a transiently-active promoter or an inducible promoter, linked to a preselected gene, the promoter and preselected gene being separated by a blocking sequence that is in turn bounded on either side by specific excision sequences, a repressible promoter operably linked to a gene encoding a site specific recombinase capable of recognizing the specific excision sequences, and a gene operably encoding a repressor specific for the repressible promoter. According to the invention, rendering the transcripts encoding the repressor protein as a template for template directed polymerization of nucleic acids, in response to some condition, e.g., provision of a viral polymerase by an infecting virus, reduces the expression of the repressor protein, thereby derepressing expression of the

recombinase, thereby causing excision of the blocking sequence, thus providing for expression of the preselected gene. In this manner, polymerase mediated inhibition of the protein coding activity of an mRNA can be used to induce the expression of a preselected gene. In one such embodiment of the invention, the organism is a plant, the repressor protein is inhibited in response to provision of a viral polymerase and the preselected gene is, for example, a lethal/toxin gene or a reporter gene.

[00084] The CRE- LOX recombinase excision system, as disclosed for example in U.S. Patent 5,723,765, has been employed for the purpose of selective recombination in numerous systems including plants and animals including mammals and their cells and is readily employed according to the present invention. Other enzymatic excision systems that can be employed according to the present invention include, for example, the resolvases, flippase, FLP, SSV1 encoded integrase, and the maize Ac/Ds transposase system (each also being disclosed in U.S. Patent 5,723,765).

[00085] A gene and a *cis*-acting, transcription-regulating DNA element ("*cis*-acting element"), such as a repressor DNA element or promoter DNA element, are considered to be linked if they occur in the same strand of DNA or within the same double stranded DNA molecule. A gene and a *cis*-acting element are considered to be operably linked if they are linked and they occur in such relative orientation and proximity that the *cis*-acting element at least partially affects the transcription of the gene. The presence of intervening DNA between the *cis*-acting element and the gene does not preclude an operable relationship.

[00086] A blocking sequence is a DNA sequence of any length that at least substantially blocks a *cis*-acting element from affecting the transcription of a gene of interest.

[00087] As further discussed herein, when the condition triggering the polymerase-mediated inactivation of the repressor is the expression of a preselected RNA transcript (or any RNA transcript having a preselected sequence or sequence region) in the cells of a multi-cellular organism, for example according to normal developmental regulation, the recombinase derepression embodiment of the invention can be used to detect expression of the preselected RNA transcript generally and to trace the cellular lineage of expression

of the preselected RNA transcript, both visibly, in real time, for example by using GFP or a derivative as a recombinase-derepressible gene, or by hybridization probing and/or histologically staining. In a further related embodiment, the derepression is performed on preselected cells in a developing embryo or tissue, for example by microinjection of a primer molecule into the cell(s) or other molecule capable of triggering the polymerase mediated inhibition of the repressor mRNA, in order to track and trace the behavior and/or lineage of the cells and their progeny. The promoter sequence that drives the expression of the preselected gene can, for example, be a constitutive promoter, a developmentally-regulated promoter, a tissue-specific promoter or an inducible promoter.

[00088] The repressor may also be a regulatory polynucleic acid that directly inhibits the activity, of a second polynucleic acid. For example, the repressor may be a catalytic polynucleic acid, such as a ribozyme or DNAzyme, engineered to cleave or otherwise degrade an mRNA transcript that encodes a peptide having, e.g., reporter activity or gene regulatory activity or cell phenotype altering activity of any sort. Here again, the cell is genetically modified so that the polynucleic acid repressor is operably linked to the activity of a viral nucleic acid polymerase, the functional interaction between the polynucleic acid molecule and the polymerase being regulated by entry of the virus into the cell.

[00089] When used to de-repress reporter genes, derepression embodiments of the invention can also be used to provide viral infection detection systems in plants and animals as described above. When used to de-repress suicide genes, e.g., lethal/toxin genes, these derepression embodiments of the invention can also be used to provide viral infection-limiting cell death in plants and animals as described above. For example, in plant embodiments of the invention lethal nucleases such as Barnase and ribonuclease A and 2 can be used and catalytic lethal proteins such as diptheria toxin and ribosomal inhibitor proteins (RIP) can be used. Use of a ribosomal inhibitor protein ("RIP") gene as a lethal gene for plants, e.g., the saponin 6 RIP, (GenBank ID SOSAPG, Accession No. X15655), is advantageous since RIP directly interferes in the expression of all protein in a plant cell, without being toxic to other organisms which ingest a plant having such cells.

[00090] Lethal/toxin genes applicable for animal cell embodiments of the invention include, but are not limited to: (a) apoptosis inducing tumor suppressor genes (e.g., p53), (b) cytotoxic genes (e.g., tumor necrosis factor, interferon-alpha), (c) suicide genes (e.g., cytosine deaminase, thymidine kinase), and (d) toxins such as pseudomonas endotoxin, ricin or diphtheria toxin subunits.

[00091] As a further example, genes coding for enzymes that convert a protoxin to a toxin can be used as the genes that are de-repressible according to the derepression embodiment. In this manner, cell death is made conditional on both the derepression of the converting enzyme and application of the protoxin to the cells/organism.

[00092] The invention is not limited to the type of cell or multi-cellular organism in which the invention is embodied or implemented. Further, cells and organisms in which the invention is embodied or implemented are within the scope of the invention. The genetic modification of cells and organisms, including complex organisms, to embody the invention, can be performed using any of the standard methods known in the art.

[00093] One embodiment of the invention comprises genetically modifying a cell or multi-cellular organism cell to render it capable of utilizing at least one preselected cell-encoded or viral polynucleic acid as a template for the template-directed polymerization of polynucleic acids by a template-directed polynucleic acid polymerase so that the activity of the polynucleic acid molecule is inhibited, wherein the cell or multi-cellular organism not so modified is at least substantially incapable of inhibiting the activity of the polynucleic molecule by utilizing it as a template for template-directed nucleic acid polymerization by a polynucleic acid polymerase. In one example of this embodiment, resistance to viral infection can be imparted to the cell or multi-cellular organism when the target polynucleic acid molecule is a viral genomic polynucleic molecule, viral replication intermediate polynucleic acid molecule, viral mRNA transcript, or other viral polynucleic acid molecule, the activity of which is required for, or contributes to, viral pathogenicity.

[00094] A further embodiment of the invention is directed to the above embodiment wherein the cell or multi-cellular organism not so genetically modified is at least substantially incapable of inhibiting the activity of the polynucleic molecule by utilizing

it as a template for template-directed nucleic acid polymerization by a polynucleic acid polymerase, even upon providing the cell or multi-cellular organism, by genetic modification or otherwise, with putative primer molecules having a 3' region of complementarity to the polynucleic acid molecule.

[00095] A still further related embodiment of the invention comprises, genetically modifying a cell or multi-cellular organism to express a template-directed polynucleic acid polymerase capable of utilizing the polynucleic acid as a template so that its activity is inhibited.

[00096] Another related embodiment further comprises genetically modifying the cell or multi-cellular organism to produce a polynucleic acid primer molecule capable of directing a template directed polynucleic acid polymerase not endogenous, i.e., not normally coded for or expressed by the cell, to utilize the polynucleic acid as a template so that its activity is inhibited. Such a polymerase includes, for example, one for which the cell is genetically engineered to express or that is provided by a virus as a result of infection of the cell by the virus.

[00097] A still further related embodiment of the invention comprises genetically modifying a cell or multi-cellular organism to express a preselected nuclease capable of degrading a target polynucleic acid molecule which has been rendered a template for a polymerase, during or after template directed nucleic acid polymerization by the polymerase, wherein the cell or multi-cellular organism otherwise lacks such a nuclease or wherein the efficiency of the degradation is increased by genetically modifying the cell or multi-cellular organism to express the preselected nuclease.

[00098] Another embodiment of the invention comprises providing the cell or multi-cellular organism with a template-directed polynucleic acid polymerase having an integral nuclease capable of degrading a preselected polynucleic acid molecule which serves as a template for the polymerase, during or after template directed nucleic acid polymerization by the polymerase, so that the activity of the preselected polynucleic acid molecule is inhibited.

[00099] <u>Examples of types of viruses for which the embodiments of the invention are generally applicable</u>

[000100] Tables 1-10 illustrate various categories of viruses and virus-like polynucleic acid molecules for which the diagnostic and viral resistance embodiments of the invention can be employed. Template-directed polymerases of, or used by, these viruses are examples of polymerases that can be used for polymerase-mediated gene regulation according to the invention. Each of the tables shows the order, family [subfamily] and genus of viruses for which the present invention can be employed. In addition, at least one type species example is provided for each genus listed. Tables 1-10 are arranged according to the following categories: Table 1 – dsDNA viruses; Table 2 – ssDNA viruses; Table 3 - DNA reverse transcribing viruses; Table 4 - RNA reverse transcribing viruses; Table 5 - dsRNA Viruses; Table 6 - negative-sense ssRNA viruses; Table 7 - positive-sense ssRNA viruses; Table 8 - naked RNA viruses; Table 9 – viroids; and Table 10 – subviral agents. It should be understood that the terms "virus" and "viruses" as used in the accompanying description and claims refers to viruses and virus-like polynucleic acids, as exemplified in Tables 1-10.

[000101] Examples of plants and their viruses for which the embodiments of the invention are applicable

[000102] The following list illustrates examples of various crop plants and their respective common viruses for which the diagnostic and viral resistance methods of the invention can be embodied: alfalfa - alfalfa mosaic, lucerene transient streak, alfalfa latent; barley - barley stripe, barley yellow dwarf, barley yellow streak, barley yellow streak mosaic, brome mosaic, oat blue dwarf; bean - bean common mosaic virus, bean yellow mosaic virus, beet curly top, cucumber mosaic virus, pea enation mosaic; beet-beet cryptic virus 1, beet cryptic virus 2, beet cryptic virus 3, beet curly top, beet mosaic, beet necrotic yellow vein, beet pseudoyellows, beet soilborne mosaic, beet western yellows, beet yellows, brassicas - cauliflower mosaic virus, turnip mosaic; capsicum species - alfalfa mosaic, beet curly top, cucumber mosaic, potato virus x, potato virus y, tobacco etch, tobacco mosaic, tobacco rattle, tomato spotted wilt; carrots - alfalfa mosaic, carrot mottle, carrot red leaf, carrot thin leaf, carrot mottley dwarf; chrysanthemum species - chrysanthemum aspermy, chrysanthemum mosaic, chrysanthemum virus b, tomato aspermy, impatiens necrotic spot; corn - maize dwarf, maize chlorotic dwarf, maize chlorotic mottle, maize dwarf, maize raydo fino, maize stripe, maize white line

mosaic; cotton - cotton leaf crumple; cucumis species - alfalfa mosaic, beet curly top, cucumber mosaic virus, lettuce infectious yellows, papaya ringspot virus, tomato spotted wilt, watermelon mosaic virus 2, squash mosaic virus, zucchini yellow mosaic; cucurbita species - beet curly top, cucumber mosaic virus, papaya ringspot virus, watermelon mosaic virus 2, squash mosaic virus, squash leaf curl, tomato spotted wilt; gladiolus- bean common mosaic virus, lily symptomless virus; lettuce - alfalfa mosaic, beet western yellows, cucumber mosaic, sowthisltle yellow vein, tobacco rattle, tobacco ringspot, tomato spotted wilt, turnip mosaic; papaya - papaya ringspot virus; pea - bean leaf roll, bean yellow mosaic virus, pea enation mosaic virus, tomato spotted wilt, pea seedborne mosaic, pea streak; peanut - peanut mottle, peanut stripe, peanut stunt, tomato spotted wilt; pepper - cucumber mosaic virus, papaya ringspot virus, watermelon mosaic virus 2, squash mosaic virus, pepper cryptic virus 1, pepper mild mottle, pepper mottle, pepper veinal mottle; potatoes - potato leaf roll, potato virus y, potato virus x, potato virus a, potato virus m, potato virus s, tobacco rattle, tomato spotted wilt; rice - rice hoja blanca; sorghum - maize dwarf mosaic, sugarcane mosaic, maize chlorotic dwarf; soybean - bean pod mottle, cowpea chlorotic mottle, peanut mottle, soybean dwarf, soybean mosaic, tobacco ringspot, tobacco streak, bean yellow mosaic, cowpea severe, peanut stripe, tobacco mosaic; strawberry - tomato ringspot, strawberry chlorosis, strawberry crinkle, strawberry latent, strawberry mottle, strawberry vein banding; sugar beets - beet curly top, beet cryptic virus 1, beet cryptic virus 2, beet cryptic virus 3, beet mosaic, beet necrotic yellow vein, beet pseudoyellows, beet soilborne mosaic, beet western yellows, beet yellows; sweet potato - sweet potato feathery mottle; tobacco - tobacco mosaic, potato virus y, tomato spotted wilt, tobacco etch, tobacco vein mottling, alfalfa mosaic; tomatoes - alfalfa mosaic, cucumber mosaic, beet curly top, tobacco etch, potato virus y, tomato bushy stunt, tomato mosaic, tomato spotted wilt, tomato ringspot, tomato mottle; and wheat - agropyron mosaic, barley stripe, barley yellow dwarf, barley yellow streak, barley yellow streak mosaic.

[000103] <u>Examples of animals (or the cells thereof) and their viruses for which the embodiments of the invention are applicable</u>

[000104] Viral pathogenic infection of agricultural animals poses a significant problem for animal health and food production. The present invention provides methods for

producing transgenic livestock and other transgenic agricultural animals that are resistant to prespecified viral pathogens. Accordingly, diagnostic and viral resistance embodiments of the invention can be employed for agricultural animals such as, but not limited to, bovids (cattle; sheep; goats, etc.), swine, fowl (chicken; quail; turkey, duck, goose, etc.); fishes, crustaceans (shrimp, crayfish, lobster, crab, etc.) and mollusca (oyster, mussel, clam, etc.).

[000105] Tables 11A and 11B show examples of various animals and their common viral pathogenic diseases for which the diagnostic and viral resistance methods of the invention can be embodied.

[000106] Aquacultured (maricultured) species have heretofore been particularly susceptible to rapid and dramatic loss as a result of viral pathogens, in part due to the typically high density of the culture conditions.

[000107] Fish viruses for which the embodiments of the invention are applicable include, but are not limited to: rhabdoviruses such as spring viremeia of carp virus; birnaviridae infections such as pancreatic necrosis virus; iridoviridae such as fish lymphocytes disease virus; salmonids with infectious hematopoietic necrocosis (IHN); viral hemorrhagic septicemia (VHS) virus; marine viral haemorrhagic septicaemia (VHS), a disease closely related to VHS known from freshwater rainbow trout farming; largemouth bass virus (LMBV); lymphocystis, a viral disease in common dab; infectious salmon anemia; Koi Herpes Virus (KHV); and Monodon baculo virus (MBV).

[000108] Shrimp viruses for which the embodiments of the invention are applicable include, but are not limited to, Infectious Hyposdermal and Hematopoietic Necrosis Virus (IHHNV); Taura Syndrome Virus (TSV); White Spot Syndrome Virus (WSSV); and Yellow Head Virus (YHV).

[000109] The genetic modification of animals to impart viral resistance against preselected viruses can also provide benefits for human health in the case of viral zoonoses, i.e., viral diseases that are transmitted to humans from animals. For example, it is well established that swine and avians, e.g., ducks, are hosts to influenza viruses and transmit such viruses to each other and to humans. Other viral pathogens are transmissible to humans from insects. According to one embodiment of the invention,

genetically modified pigs embodying resistance to influenza virus according to the invention are raised, thereby reducing zoonetic transmission of the disease to the human population.

[000110] Embodiment related to transplantation of cells genetically modified to embody the invention

[000111] One embodiment of the invention provides a method of making a subject mammal resistant to pathogencity of a preselected viral pathogen to which the organism is ordinarily susceptible, comprising the steps of: providing genetically-modified cells capable of integrating with a tissue of the subject mammal, the tissue ordinarily being susceptible to the viral pathogen, wherein (i) the modification causes the activity of at least one preselected viral polynucleic acid molecule of the virus to be diminished in the cells as a result of the preselected viral polynucleic acid molecule being rendered a template for the template directed polymerization of nucleic acid molecules within the cell; or (ii) the modification causes the activity of at least one preselected cellular RNA transcript to be diminished in response to infection by the virus as a result of the preselected cellular transcript being rendered a template for the template directed polymerization of nucleic acid molecules within the cell, resulting in (a) resistance of the cell and/or (b) resistance of the multi-cellular organism as a whole to the pathogenicity of the virus by reducing the replication and/or spread of the virus within the subject; and introducing the genetically modified cells into the subject mammal.

[000112] A related embodiment of the invention provides that the mammal is already infected with the virus when the genetically modified cells are introduced into the mammal.

[000113] A further, related embodiment provides that the step of providing the genetically modified cells further comprises first isolating unmodified cells from the tissue of the subject mammal, then modifying these cells as described, and thereafter introducing the so-modified cells back into the subject mammal.

[000114] Further embodiments of the above-described methods provide that the subject mammal is a human being and (i) the tissue comprises lymphoid cells or lymphoid cell precursors and the virus is a human immunodeficiency virus, such as HIV-1 or HIV-2; or

(ii) the tissue comprises hepatic cells or hepatic cell precursors and the virus is a Hepatitis virus, for example, Hepatitis B or Hepatitis C.

[000115] Another related embodiment of the invention provides genetically-modified cells capable of integrating with a tissue of a subject mammal, the tissue ordinarily being susceptible to a viral pathogen, wherein (i) the modification causes the activity of at least one preselected viral polynucleic acid molecule of the virus to be diminished in the cells as a result of the preselected viral polynucleic acid being rendered a template for the template directed polymerization of nucleic acid molecules within the cell; or (ii) the modification causes the activity of at least one preselected cellular RNA transcript to be diminished in response to infection by the virus as a result of the preselected cellular transcript being rendered a template for the template directed polymerization of nucleic acid molecules within the cell, resulting in (a) resistance of the cell to the virus and/or (b) resistance of the mammal as a whole to the pathogenicity of the virus by reducing the replication and/or spread of the virus within the subject upon integration of the genetically modified cells into the subject mammal.

[000116] RNA silencing

[000117] In a first variation of the cell-based and multi-cellular organism-based embodiments of the present invention, the cells constituting the elements of the invention are not capable of RNA silencing in response to long dsRNA or long RNA/cDNA hybrid molecules and the rendering of a preselected RNA molecule to be template for a preselected polynucleic acid polymerase to reduce the "normal" activity of the polynucleic acid molecule forms a long dsRNA molecule or long RNA/cDNA hybrid which does not trigger RNA silencing of polynucleic acid molecules in the cell.

[000118] In a related variation of the invention, the cells constituting the elements of the invention are not capable of RNA silencing in response to long dsRNA or long RNA/cDNA hybrid molecules but are capable of RNA silencing in response to siRNAs or similar small RNA/cDNA hybrid molecules. Various mammalian cells including human cells have this characteristic. In a related subvariation of the invention, such cells are made to express a member of the RNAse III family, such as Dicer, to cause the long dsRNA or long RNA/cDNA hybrid molecules formed by rendering the preselected RNA

molecule to be a template for a preselected polynucleic acid polymerase to reduce the activity of the polynucleic acid molecule to be processed into siRNAs or small RNA/cDNA hybrids which trigger RNA silencing of polynucleic acid molecules in the cell.

[000119] In a further variation of the invention, the cells constituting the elements of the invention are incapable of RNA silencing in response to long dsRNA or long RNA/cDNA hybrid molecules, whether or not such molecules are processed by an RNAse III enzyme, and the rendering of a preselected RNA molecule to be a template for a preselected polynucleic acid polymerase to reduce the activity of the preselected polynucleic acid molecule forms such a long dsRNA molecule or long RNA/cDNA hybrid. In one embodiment of this variation, an RNAse III enzyme is expressed in the cell(s), for example, by genetically-modifying the cell(s) to express the enzyme, and the RNAse III processes the long dsRNA molecule or long RNA/cDNA hybrid but RNA silencing is not triggered in response. In accordance with the described role of RISC in RNA silencing, such a cell can be created by genetically modifying an otherwise RNAi-RNA silencing-competent cell to knock out, for example by homologous recombination, one or more components of the RISC complex.

[000120] In another variation of the invention, the cells constituting the elements of the invention are capable of RNA silencing in response to long dsRNA or long RNA/cDNA hybrid molecules and the rendering of a preselected RNA molecule to be a template for a preselected polynucleic acid polymerase, to reduce the activity of the preselected polynucleic acid molecule, forms such a long dsRNA molecule or long RNA/cDNA hybrid which further triggers RNA silencing of polynucleic acid molecules in the cell.

[000121] When RNA silencing is triggered by rendering the preselected polynucleic acid molecule(s) a template in the cells embodying the invention, the polynucleic acid molecules silenced may be other copies of the same preselected polynucleic acid molecule which may be present in the cell, similar polynucleic acid molecules encoded by the same gene or different polynucleic acid molecules encoded by different genes but which have regions of complementarity with at least one strand of the siRNA fragment(s)

generated from the preselected polynucleic acid molecule which is rendered as a template.

[000122] In a related variation of the invention, the cells constituting the elements of the invention are capable of RNA silencing in response to long dsRNA but not in response to long RNA/cDNA hybrid molecules. In this variation, use of a reverse transcriptase to render a preselected RNA molecule to be template for a preselected polynucleic acid polymerase to reduce the activity of the preselected polynucleic acid molecule creates a long RNA/cDNA hybrid which does not trigger said RNA silencing, while use of a RNA-dependent RNA polymerase creates a long dsRNA which does trigger said RNA silencing.

[000123] In another related variation of the invention, the cells constituting the elements of the invention are capable of RNA silencing in response to long RNA/cDNA hybrids but a reverse transcriptase having an integral or closely-associated RNAse H is used as the polymerase which renders the preselected polynucleic acid molecule as a template for template directed polymerization to reduce the activity of the preselected polynucleic acid molecule so that a long RNA/cDNA hybrid is not formed as a result of said polymerization. In a similar related variation, the RNAse H is not integral or closely associated with the reverse transcriptase, but is nevertheless expressed in the cell and active against the RNA template component of the RNA/cDNA hybrid being formed by the reverse trascriptase. In either case, the RNAse H hydrolyzes the RNA template strand as the cDNA is synthesized so that formation of a long RNA/cDNA hybrid molecule competent for RNA silencing is at least substantially not formed and RNA silencing is at least substantially prevented.

[000124] According to the invention, any gene of interest may be placed under the direct or indirect control of any operable transcriptional repressor or activator by genetically modifying a cell (stably or transiently) and the repression or activation of the expression of the gene of interest can be selectively modulated, according to the invention, by (i) rendering the mRNA transcripts coding for the transcriptional regulator protein, or by (ii) rendering an RNA molecule which is itself the transcriptional regulator (for example, a ribozyme engineered to cleave the transcript of the gene of interest), as

targets of an RNA silencing mechanism in response to a prespecified condition, i.e., a particular stimulus or triggering event. The RNA silencing embodiments of the invention that affect gene expression and/or alter genotype in a detectable manner in response to the presence in the cell of particular polynucleic acid sequences, for example, those that occur in a cell as a result of viral infection of the cells by particular viruses, also generally provide assays and test cells and organisms for the detection of such sequences, and their vectors if any, in a test sample, in the environment and/or in an organism comprising such test cells. Such assays can be conducted, for example, by contacting test cells with samples, optionally also using positive and/or negative controls, and by determining the extent to which the sample triggers the condition-specific, RNA silencing-mediated gene expression and/or genotypic changes, with or without comparison to any controls.

[000125] In one RNA silencing embodiment of the invention, an RNA molecule is rendered a target of RNA silencing in response to a prespecified condition and is an mRNA coding for a transcriptional activator protein or a transcriptional repressor protein that activates or represses, respectively, the expression of one or more other preselected genes of any sort including, but not limited to: reporter genes, lethal/toxic genes, essential genes, cell phenotype altering genes, herbicide resistance genes, and genes which themselves code for a transcriptional regulatory protein controlling the expression of still one or more other genes. Further, the invention provides that the preselected genes can be of the sort found in nature under the control of the regulatory protein or can be genes not found in nature under control of the particular regulatory protein.

[000126] In a related RNA silencing embodiment of the invention, an RNA molecule is rendered a target of RNA silencing in response to a prespecified condition and is an mRNA coding for a transcriptional repressor protein which represses the expression, or is engineered to repress the expression of, one or more other preselected genes such as, but not limited to, a reporter gene or a suicide gene which causes an infected cell to die, thus limiting spread of the viral infection within a multi-cellular organism or to other organisms.

[000127] Another RNA silencing related embodiment of the invention provides introducing a series of sequences into a cell or multi-cellular organism that includes an

excisable sequence element that is in turn bounded on either side by specific excision sequences, a repressible promoter operably linked to a gene encoding a site specific recombinase capable of recognizing the specific excision sequences, and a gene operably encoding a repressor specific for the repressible promoter. According to the invention, selectively triggering an RNA silencing cascade against the repressor, e.g., in response to some condition, reduces the expression of the repressor protein, thereby derepressing expression of the recombinase, thereby causing excision of the excisable sequence, thus altering the sequence of the DNA molecule in which the excisable sequence was previously found. In this manner, RNA silencing of the protein coding activity of a preselected mRNA can be used to induce a genotypic change in a cell. The presence or absence of the genotypic change in the cell or within a population of such cells can be detected by standard molecular biological methods. The resulting genotypic changes present in the cell and its progeny if any can also serve as a record of exposure to the condition which caused the genotypic change to occur, for example, infection of the cell by particular virus(es) or the presence of any preselected RNA sequence in the cell.

[000128] In a related embodiment of the invention, the excisable sequence element comprises an expression cassette comprising at least one gene of interest, and transcriptional regulatory elements providing for expression of the gene in a constitutive or regulated manner (developmentally, environmentally, or inducibly). In this embodiment, when recombinase expression is de-repressed, as described above, the excisable element is deleted and expression of the at least one gene of interest from the cassette is eliminated or the possibility of regulative expression of the gene is eliminated as a result of deleting the cassette.

[000129] Another RNA-silencing related embodiment provides introducing a series of sequences into a cell or multi-cellular organism that includes a promoter, such as a constitutively-active promoter, a transiently-active promoter or an inducible promoter, linked to a preselected gene the promoter and preselected gene being separated by a blocking sequence that is in turn bounded on either side by specific excision sequences, a repressible promoter operably linked to a gene encoding a site specific recombinase capable of recognizing the specific excision sequences, and a gene operably encoding a repressor specific for the repressible promoter. According to the invention, selectively

triggering an RNA cascade against the repressor protein, e.g., in response to some condition, renders the transcripts encoding the repressor protein as a template for template directed polymerization of nucleic acids, in response to some condition, e.g., provision of a viral polymerase by an infecting virus, and reduces the expression of the repressor protein, thereby derepressing expression of the recombinase, thereby causing excision of the blocking sequence, thus providing for expression of the preselected gene. In this manner, inactivation of the protein coding activity of an mRNA via an RNA silencing cascade can be used to induce the expression of a preselected gene. In one such embodiment of the invention, the organism is a plant, the repressor protein is inhibited in response to viral infection and the preselected gene is, for example, a lethal/toxin gene or a reporter gene or an antiviral gene, for example, a gene coding for RNA silencing-inducing short RNA hairpins specific for the inhibition of viral polynucleic acid molecules or cellular polynucleic acid molecules in the cell.

[000130] According to the embodiments of the invention in which an mRNA transcript coding for a transcriptional regulatory protein, or a transcription-regulating RNA molecule, is targeted by RNA silencing in response to some stimuli, the stimuli may be of any sort including, but not limited to, the following cases:

[000131] the stimuli comprises (i) contacting the cell with a prepared, e.g., synthetic, siRNA at least one of the strands of the siRNA being at least substantially complementary to the mRNA transcript (or transcription-regulating RNA molecule) or (ii) providing the cell with expression of such an siRNA;

[000132] the stimuli comprises contacting the cell with a short RNA hairpin molecule wherein at least one of the complementary segments of the double stranded stem structure is at least substantially complementary to the mRNA transcript (or transcription-regulating RNA molecule), or providing the cell with expression of such a molecule;

[000133] the stimuli comprises providing the cell with long dsRNA which is processed into siRNAs, the siRNAs having at least one strand having at least substantial complementarity with the mRNA molecule (or transcription-regulating RNA molecule);

[000134] the stimuli comprises rendering the mRNA molecule (or transcription-regulating RNA molecule) initially as a template for the template directed polymerization

of nucleic acids by a polymerase, wherein siRNAs or small RNA/cDNA hybrids are produced from the double stranded polymerization product, said siRNAs or small RNA/cDNA hybrids causing RNA silencing of other of the same mRNA molecules coding for the regulatory protein (or other of the same transcription-regulating RNA molecules);

[000135] the stimuli comprises expression of an RNA transcript, e.g., an mRNA transcript under control of developmentally regulated promoters and/or other transcriptional regulatory elements, (i) the cell being capable (genetically modified if necessary) of rendering the transcript, upon its expression, as a nucleic acid polymerization template to form a long dsRNA or long RNA/cDNA molecule which triggers RNA silencing, or (ii) the cell expressing a long single stranded RNA or DNA molecule at least substantially complementary to the RNA transcript, wherein, upon expression of the RNA transcript, the transcript and the expressed complementary molecule hybridize to form an at least partially double stranded molecule capable of triggering RNA silencing or (iii) the RNA transcript being itself capable of triggering RNA silencing, for example, the transcript being or comprising an RNA silencing-competent RNA hairpin;

[000136] the stimuli comprises infection of the cell by a virus and the virus characteristically forms long dsRNA which triggers RNA silencing in response to the infection, which RNA silencing comprises production of siRNAs wherein at least one strand is at least substantially complementary to the mRNA transcript for the transcriptional regulatory protein (or transcription-regulating RNA molecule), for example, as a result of genetically engineering the gene for the mRNA transcript (or transcription-regulating RNA molecule) to contain at least one region of homology to the characteristic long dsRNA region of the virus ;

[000137] the stimuli comprises infection of the cell by a virus and the cell is genetically modified to render an mRNA transcript of the virus or another polynucleic acid molecule of the virus as a template for template directed polymerization of nucleic acids so that a long dsRNA or long RNA/cDNA is formed, the long molecule triggering RNA silencing in response to the infection, which RNA silencing comprises production of siRNAs

wherein at least one strand is at least substantially complementary to the mRNA transcript for the transcriptional regulatory protein (or transcription-regulating RNA molecule); and

[000138] the stimuli comprises the presence in a cell, e.g., by the deliberative or chance introduction in or production within the cell, of a single ssRNA or ssDNA molecule, the molecule having at least one region of known sequence, wherein the cell expresses (naturally or as a result of genetic engineering) an ssRNA or ssDNA molecule having a region of at least substantial complementarity to the region of known sequence, so that the introduced or produced single stranded molecule and the cellularly expressed single stranded molecule hybridize to form either a dsRNA molecule or RNA:DNA hybrid molecule capable of triggering RNA silencing, directly or transitively, against the mRNA molecule encoding the transcriptional regulator protein (or the transcription-regulating RNA molecule). The mRNA molecule encoding the transcriptional regulator protein (or the transcription-regulating RNA molecule) may itself be one of one or more cellularly expressed single stranded molecule having a region of at least substantial complementarity to the region of known sequence.

[000139] In a related variation of the invention, the mRNA molecule encoding the transcriptional regulator protein (or the transcription-regulating RNA molecule) does not have substantial complementarity with the region of known sequence or even with any of the sequence of the introduced or produced single stranded molecule, but is transitively targeted by RNA silencing, for example, by virtue of sequence shared with or complementary to sequence in the cellularly expressed single stranded molecule that does have a region at least substantially complementarity to the introduced or produced single stranded molecule or by further degrees of transitive silencing mediated by further intermediate polynucleic molecules.

[000140] An example of chance introduction or production of a ssRNA or ssDNA molecule in a cell, or cell of an organisms occurs when particular viruses, virus like agents or other environmental polynucleic acid molecules infect or otherwise enter or are produced in cells as a result of exposure to these agents in the environment.

[000141] In the above examples, when the sequence of at least a part of the long dsRNA or long RNA/cDNA molecule is known (such as when the trigger is a known virus or cellular transcript), the gene coding for the transcriptional regulatory protein (or transcription-regulating RNA molecule), can, if necessary, be modified to contain regions of homology to one or both of the strands of the triggering long dsRNA or long RNA/cDNA molecule so that the siRNA in conjunction with RISC will target these regions using siRNAs formed from the long double stranded molecules. Conversely, if the polynucleic acid molecule for which it is desired to trigger RNA silencing upon expression of the molecule is encoded by a cellular gene (endogenous or transgene), the sequence of the gene for this molecule can, if necessary, be modified to comprise regions of homology to the mRNA transcript for the transcriptional regulatory protein (or to the transcription-regulating RNA molecule) so that upon the polynucleic acid molecule becoming double stranded it is processed to render siRNAs wherein at least one strand is at least substantially complementary to the target repressor protein transcript (or transcription-regulating RNA molecule).

[000142] Generally, where RNA silencing is operable, any first molecule that will be targeted to produce siRNAs can be modified to induce RNA silencing against a selected downstream molecule by modifying the first molecule (e.g. by modifying the gene expressing the molecule) to comprise sequence that will form siRNA strands having homology with or being complementary to the sequence of the selected downstream molecule. Alternatively, or in addition, a selected molecule can be rendered as a downstream target of the first molecule by modifying the second molecule (e.g. by modifying the gene expressing the molecule) so that it comprises at least one sequence element having homology with or being complementary to the sequence of the first molecule that forms siRNAs. For an mRNA molecule, such modification may be made, for example, in the 5' untranslated region, the 3' untranslated region, in naturally occurring or genetically engineered intronic sequences, and/or even in the protein coding sequence so long as any desired protein coding activity and corresponding protein activity is not negatively impacted to a substantial extent.

[000143] Transitive RNA silencing embodiments corresponding to the direct RNA silencing embodiments of the invention can also be provided, when desired, according to

the invention. For example, those skilled in the art will readily appreciate that, in organisms and cell types where transitive RNA silencing is operable, at least one intermediate polynucleic acid molecule may be used to indirectly mediate the transfer of the RNA silencing signal, via siRNAs, from an initial, or otherwise "upstream", double-stranded, long triggering molecule to the target mRNA transcript of the transcriptional regulatory protein. In this case the intermediate molecule will have complementary sequences to at least one siRNA strand produced from the initial molecule, thereby rendering it a target for siRNAs formed from the initial molecule, and at least one other sequence, generally in the three-prime direction with respect to the primer strand of said siRNA, the other sequence being complementary or having homology to the ultimate target RNA transcript in question so that formation of a long dsRNA from the intermediate molecule causes the formation of siRNAs wherein at least one strand is complementary to the ultimate target. Thus, when it is known that a first molecule will be subject to RNA silencing, the silencing event can be transitively targeted to one or more selected other molecules by providing the cell with expression of one or more intermediate molecules.

[000144] In cells or organisms in which the siRNA in conjunction with RISC or other factors functions to prime the target molecule and induce template directed polymerization of nucleic acids using the target as a template, the introduction of the regions of homology is an introduction of a PBS according to the invention and, likewise, providing an siRNA is an example of providing a primer molecule.

[000145] The invention also encompasses the case where the siRNA in conjunction with RISC or other factors does not induce, or is not capable of inducing template directed polymerization of nucleic acid molecules but nevertheless binds to the target and optionally causes nuclease activity against the target.

[000146] As further discussed herein, when the condition triggering the polymerase-mediated inactivation of the repressor is the expression of a preselected RNA transcript (or any RNA transcript having a preselected sequence) in the cells of a multi-cellular organism according to normal developmental regulation, the recombinase derepression embodiment of the invention can be used to detect expression of the preselected RNA

transcript generally and to trace the cellular lineage of expression of the preselected RNA transcript, both visibly, in real time, for example by using GFP or a derivative as a recombinase-derepressible gene, or by histological analysis.

[000147] The techniques used for producing genetically-engineered cells and transgenic multi-cellular organisms according to the invention are routine in the art and are, accordingly, only briefly described herein.

[000148] As referred to herein, a genetically modified cell or multi-cellular organism refers to a cell or organism that has been genetically engineered to embody genetic sequences required for the functioning of the invention, or which are derived from such a cell or organism and comprise the introduced change(s). Genetically modified cells and genetically –modified organisms according to the invention include cells and organisms genetically engineered to contain transgenes and/or to have genetic sequence alterations including deletions, or which are derived from such a cell or organism and comprise the introduced change(s). Cells transiently transformed with DNA or RNA constructs are also within the scope of the invention.

[000149] A variety of routine methods for introducing DNA into cells for the introduction of transgenes to the cells and/or to effectuate other genetic changes in the cells are well established. Predetermined deletions and other sequence changes of preselected genes and other genetic sequences in a cell can be performed using homologous recombination techniques as known in the art.

[000150] Transgenic animals are animals having cells that contain a transgene, wherein the transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic, stage. Non-human animals into which transgenes or other discrete genetic changes can be introduced by genetic engineering techniques known in the art include, but are not limited to, mice, goats, sheep, pigs, cows and other domestic farm animals, as well as fish, birds, and crustaceans and insects. A transgenic animal can be created, for example, by introducing a nucleic acid sequence encoding a protein of interest (typically linked to appropriate regulatory elements, such as a constitutive or tissue-specific promoter and/or other regulatory elements) into the male pronuclei of a fertilized oocyte, e.g., by microinjection, and allowing the oocyte to develop in a

pseudopregnant female foster animal. An alternative method comprises introducing a desired DNA into the genome an embryonic stem cell and regenerating the organism by introducing the modified stem cell into a developing early-stage embryo. A transgenic founder animal can be used to breed additional animals carrying the transgene.

[000151] Techniques for transforming a wide variety of higher plant species are also well established and described in the technical and scientific literature. A DNA sequence coding for a desired gene product can be combined with transcriptional and translational initiation regulatory sequences that will direct the transcription of the sequence from the gene in the intended tissues of the transformed plant.

[000152] For example a plant promoter fragment may be employed which will direct expression of the gene in all or substantially all tissues of a regenerated plant. Such promoters are referred to herein as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation. Examples of constitutive promoters include the cauliflower mosaic virus (CaMV) 35S transcription initiation region, the 1'- or 2'- promoter derived from T-DNA of *Agrobacterium tumefaciens*, and other transcription initiation regions from various plant genes known to those of skill.

[000153] Alternatively, the plant promoter may direct expression of a nucleic acid of the invention in a specific tissue, organ or cell type (i.e. tissue-specific promoters) or may be otherwise under more precise environmental or developmental control (e.g., inducible promoters). Examples of environmental conditions that may effect transcription by inducible promoters include anaerobic conditions, elevated temperature, the presence of light, or application of chemicals such as hormones. Exemplary promoters for this purpose include promoters from glucocorticoid receptor genes (Aoyama and Chua, Plant J 11:605-12 (1997)). Tissue-specific promoters may only promote transcription within a certain time frame of developmental stage within that tissue. Other tissue specific promoters may be active throughout the life cycle of a particular tissue.

[000154] Techniques for the production of transgenic plants are well established. For example, the DNA construct comprising a transgene may be introduced directly into the genomic DNA of the plant cell using techniques such as electroporation and

microinjection of plant cell protoplasts, or the DNA constructs can be introduced directly to plant tissue using ballistic methods, such as DNA particle bombardment, or *Agrobacterium* tumefaciens-mediated transformation techniques, including disarming and use of binary vectors, are well described in the scientific literature. Transformed plant cells that are derived by any of the above transformation techniques can be cultured to regenerate a whole plant that possesses the transformed genotype and thus the desired phenotype such as increased seed mass. Regeneration can also be obtained from plant callus, explants, organs, or parts thereof.

[000155] It should be understood that the above descriptions are meant to be illustrative. With respect to its cell-based embodiments, the invention is not limited by the subcellular compartment(s) in which its components are processed and ultimately function. Many embodiments within the scope of the invention may be apparent to those of skill in the art upon reviewing the above descriptions. The scope of the invention should, therefore, be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled.

[000132] **Table 1 – dsDNA Viruses**

Order	Family [Subfamily]	Genus	Type Species Example
Caudovirales	Myoviridae	"T4-like viruses"	Enterobacteria phage T4
		"P1-like viruses"	Enterobacteria phage P1
		"P2-like viruses"	Enterobacteria phage P2
		"Mu-like viruses"	Enterobacteria phage Mu
		"SPO1-like viruses"	Bacillus phage SPO1
		"ΦH-like viruses"	Halobacterium virus ΦH
	Siphoviridae	"λ-like viruses"	Enterobacteria phage λ
		"T1-like viruses"	Enterobacteria phage T1
		"T5-like viruses"	Enterobacteria phage T5
		"c2-like viruses"	Lactococcus phage c2
		"L5-like viruses"	Mycobacterium phage L5
		"ψM1-like viruses"	Methanobacterium ψM1
	Podoviridae	"T7-like viruses"	Enterobacteria phage T7
		"φ29-like viruses"	Bacillus phage φ29
		"P22-like viruses"	Enterobacteria phage P22
	Rudiviridae	Rudivirus	Sulfolobus virus SIRV1
	Tectiviridae	Tectivirus	Enterobacteria phage PRD1
	Corticoviridae	Corticovirus	Alteromonas phage PM2
	Lipothrixviridae	Lipothrixvirus	Thermoproteus virus 1
	Plasmaviridae	Plasmavirus	Acholeplasma phage L2
	Fuselloviridae	Fusellovirus	Sulfolobus virus SSV1

Order	Family [Subfamily]	Genus	Type Species Example
	Phycodnaviridae	Chlorovirus	Paramecium bursaria Chlorella virus 1
		Prasinovirus	Micromonas pusilla virus SP'1
		Prymnesiovirus	Chrysochromulina brevifilum virus PW1
		Phaeovirus	Ectocarpus siliculosis virus 1
		"Sulfolobus SNDV-like viruses"	Sulfolobus virus SNDV
	Poxviridae [Chordopoxvirinae]	Orthopoxvirus	Vaccinia virus
		Parapoxvirus	Orf virus
		Avipoxvirus	Fowlpox virus
		Capripoxvirus	Sheeppox virus
		Leporipoxvirus	Myxoma virus
		Suipoxvirus	Swinepox virus
		Molluscipoxvirus	Molluscum contagiosum virus
		Yatapoxvirus	Yaba monkey tumor virus
	[Entomopoxvirinae]	Entomopoxvirus A	Melolontha melolontha entomopoxvirus
		Entomopoxvirus B	Amsacta moorei entomopoxvirus
		Entomopoxvirus C	Chironomus luridus entomopoxvirus
	Iridoviridae	Iridovirus	Invertebrate iridescent virus 6
		Chloriridovirus	Invertebrate iridescent virus 3
		Ranavirus	Frog virus 3
		Lymphocystivirus	Lymphocystis disease virus 1
	Polydnaviridae	Ichnovirus	Campoletis sonorensis ichnovirus
		Bracovirus	Cotesia melanoscela bracovirus
	Herpesviridae	Ictalurivirus (was "Ictalurid herpes-like viruses")	Ictalurid herpesvirus 1
	[Alphaherpesvirinae]	Simplexvirus	Human herpesvirus 1

Order	Family [Subfamily]	Genus	Type Species Example
		Varicellovirus	Human herpesvirus 3
		Mardivirus (was "Marek's disease-like viruses")	Gallid herpesvirus 2
		Iltovirus (was "Infectious laryngo-tracheitis-like viruses")	Gallid herpesvirus 1
	[Betaherpesvirinae]	Cytomegalovirus	Human herpesvirus 5
		Muromegalovirus	Murid herpesvirus1
		Roseolovirus	Human herpesvirus 6
	[Gammaherpesvirinae]	Lymphocryptovirus	Human herpesvirus 4
		Rhadinovirus	Saimiriine herpesvirus 2
	Polyomaviridae	Polyomavirus	Simian virus 40
	Papillomaviridae	Papillomavirus	Cottontail rabbit papillomavirus
	Adenoviridae	Mastadenovirus	Human adenovirus C
		Aviadenovirus	Fowl adenovirus A
		Atadenovirus	Ovine adenovirus D
		Siadenovirus	Turkey adenovirus B
	Ascoviridae	Ascovirus	Spodoptera frugiperda ascovirus
	Baculoviridae	Nucleopolyhedrovirus	Autographa californica nucleopolyhedrovirus
		Granulovirus	Cydia pomonella granulovirus
	Nimaviridae	Whispovirus	White spot syndrome virus 1
	Asfarviridae	Asfivirus	African swine fever virus
		Rhizidiovirus	Rhizidiomyces virus

[000133] **Table 2 – ssDNA Viruses**

Order	Family [Subfamily]	Genus	Type Species Example
	Inoviridae	Inovirus	Enterobacteria phage M13
		Plectrovirus	Acholeplasma phage MV-L51
	Microviridae	Microvirus	Enterobacteria phage φX174
		Spiromicrovirus	Spiroplasma phage 4
		Bdellomicrovirus	Bdellovibrio phage MAC1
		Chlamydiamicrovirus	Chlamydia phage 1
	Geminiviridae	Mastrevirus	Maize streak virus
		Curtovirus	Beet curly top virus
		Begomovirus	Bean golden mosaic virus - Puerto Rico
	Circoviridae	Circovirus	Porcine circovirus
		Gyrovirus	Chicken anaemia virus
	Nanoviridae	Nanovirus	Subterranean clover stunt virus
		Babuvirus	Babana bunchy top virus
	Parvoviridae [Parvovirinae]	Parvovirus	Murine Minute virus
		Erythrovirus	B19 virus
		Dependovirus	Adeno-associated virus 2
	[Densovirinae]	Densovirus	Junonia coenia densovirus
		Iteravirus	Bombyx mori densovirus
		Brevidensovirus	Aedes aegypti densovirus

[000134] **Table 3 – DNA Reverse Transcribing Viruses**

Order	**Family**	**Genus**	**Type Species Example**
	Pseudoviridae	Pseudovirus	Saccharomyces cerevisiae Ty1 virus
		Hemivirus	Drosophila melanogaster copia virus
	Metaviridae	Metavirus	Saccharomyces cerevisiae Ty3 virus
		Errantivirus	Drosophila melanogaster gypsy virus
	Hepadnaviridae	Orthohepadnavirus	Hepatitis B virus
		Avihepadnavirus	Duck hepatitis B virus
	Caulimoviridae	Badnavirus	Commelina yellow mottle virus
		Caulimovirus	Cauliflower mosaic virus
		Tungrovirus (was "Rice tungro bacilli-form-like viruses")	Rice tungro bacilliform virus
		Soymovirus (was "Soybean chlorotic mottle-like viruses")	Soybean chlorotic mottle virus
		Cavemovirus (was "Cassava vein mosaic-like viruses")	Cassava vein mosaic virus
		Petuvirus (was "Petunia vein clearing-like viruses")	"Petunia vein clearing virus

[000135] **Table 4 – RNA Reverse Transcribing Viruses**

Order	**Family [Subfamily]**	**Genus**	**Type Species Example**
	Retroviridae [Orthoretrovirinae]	Alpharetrovirus	Avian leucosis virus
		Betaretrovirus	Mouse mammary tumour virus
		Gammaretrovirus	Murine leukeamia virus
		Deltaretrovirus	Bovine leukaemia virus
		Epsilonretrovirus	Walleye dermal sarcoma virus
		Lentivirus	Human immunodeficiency virus 1
	[Spumaretrovirinae]	Spumavirus	Simian foamy virus

[000136] **Table 5 – dsRNA Viruses**

Order	**Family**	**Genus**	**Type Species Example**
	Cystoviridae	Cystovirus	Pseudomonas phage ψ6
	Reoviridae	Orthoreovirus	Mammalian orthoreovirus
		Orbivirus	Bluetongue virus
		Rotavirus	Rotavirus A
		Coltivirus	Colorado tick fever virus
		Seadornavirus	Kadipiro virus
		Aquareovirus	Aquareovirus A
		Cypovirus	Cypovirus 1
		Entomoreovirus	Hyposoter exiguae reovirus
		Fijivirus	Fiji disease virus
		Phytoreovirus	Rice dwarf virus
		Oryzavirus	Rice ragged stunt virus
	Birnaviridae	Aquabirnavirus	Infectious pancreatic necrosis virus
		Avibirnavirus	Infectious bursal disease virus
		Entomobirnavirus	Drosophila X virus
	Totiviridae	Totivirus	Saccharomyces cerevisiae virus L-A
		Giardiavirus	Giardia lamblia virus
		Leishmaniavirus	Leishmania RNA virus 1-1
	Chrysoviridae	Chrysovirus	Penicillium chrysogenum virus
	Partitiviridae	Partitivirus	Atkinsonella hypoxylon virus
		Alphacryptovirus	White clover cryptic virus 1
		Betacryptovirus	White clover cryptic virus 2
	Hypoviridae	Hypovirus	Cryphonectria hypovirus 1-EP713
		Varicosavirus	Lettuce big-vein virus

[000137] **Table 6 – Negative Sense ssRNA Viruses**

Order	Family	Genus	Type Species Example
Mononegavirales	Paramyxoviridae [Paramyxovirinae]	Respirovirus	Sendai virus
		Morbillivirus	Measles virus
		Rubulavirus	Mumps virus
		Henipavirus	Hendra virus
		Avulavirus	Newcastle disease virus
	[Pneumovirinae]	Pneumovirus	Human respiratory syncytial virus
		Metapneumovirus	Avian pneumovirus
	Rhabdoviridae	Vesiculovirus	Vesicular stomatitis Indiana virus
		Lyssavirus	Rabies virus
		Ephemerovirus	Bovine ephemeral fever virus
		Cytorhabdovirus	Lettuce necrotic yellows virus
		Nucleorhabdovirus	Potato yellow dwarf virus
		Novirhabdovirus	Infectious hematopoietic necrosis virus
Mononegavirales	Filoviridae	Marburgvirus (was "Marburg-like viruses")	Lake Victoria marburgvirus (was Marburgvirus)
		Ebolavirus (was "Ebola-like viruses")	Zaire ebolavirus (was Zaire Ebola virus)
	Bornaviridae	Bornavirus	Borna disease virus
	Orthomyxoviridae	Influenzavirus A	Influenza A virus
		Influenzavirus B	Influenza B virus
		Influenzavirus C	Influenza C virus
		Thogotovirus	Thogoto virus
		Isavirus	Infectious salmon anemia virus
	Bunyaviridae	Orthobunyavirus	Bunyamwera virus
		Hantavirus	Hantaan virus
		Nairovirus	Dugbe virus
		Phlebovirus	Rift Valley fever virus

Order	Family	Genus	Type Species Example
		Tospovirus	Tomato spotted wilt virus
	Arenaviridae	Arenavirus	Lymphocytic choriomeningitis virus
		Ophiovirus	Citrus psorosis virus
		Tenuivirus	Rice stripe virus
		Deltavirus	Hepatitis delta virus

[000138] **Table 7 – Positive Sense ssRNA Viruses**

Order	Family	Genus	Type Species Example
	Leviviridae	Levivirus	Enterobacteria phage MS2
		Allolevivirus	Enterobacteria phage Qβ
	Dicistroviridae	Cripavirus	Cricket paralysis virus
		Iflavirus (was "Infectious flacherie-like viruses")	Infectious flacherie virus
	Picornaviridae	Enterovirus	Poliovirus
		Rhinovirus	Human rhinovirus A
		Hepatovirus	Hepatitis A virus
		Cardiovirus	Encephalomyocarditis virus
		Aphthovirus	Foot-and-mouth disease virus
		Parechovirus	Human parechovirus
	Sequiviridae	Sequivirus	Parsnip yellow fleck virus
		Waikavirus	Rice tungro spherical virus
	Comoviridae	Comovirus	Cowpea mosaic virus
		Fabavirus	Broad bean wilt virus 1
		Nepovirus	Tobacco ringspot virus
	Potyviridae	Potyvirus	Potato virus Y
		Rymovirus	Ryegrass mosaic virus
		Bymovirus	Barley yellow mosaic virus
		Macluravirus	Maclura mosaic virus
		Ipomovirus	Sweet potato mild mottle virus
		Tritimovirus	Wheat streak mosaic virus
	Caliciviridae	Vesivirus	Swine vesicular exanthema virus
		Lagovirus	Rabbit hemorrhagic disease virus

Order	**Family**	**Genus**	**Type Species Example**
		Norovirus (was "Norwalk-like viruses")	Norwalk virus
		Sapovirus (was "Sapporo-like viruses")	Sapporo virus
	Hepeviridae	Hepevirus (was "Hepatitis E-like viruses")	Hepatitis E virus
	Astroviridae	Astrovirus	Human astrovirus 1
	Nodaviridae	Alphanodavirus	Nodamura virus
		Betanodavirus	Striped jack nervous necrosis virus
	Tetraviridae	Betatetravirus	Nudaurelia capensis β virus
		Omegatetravirus	Nudaurelia capensis ω virus
	Tombusviridae	Tombusvirus	Tomato bushy stunt virus
		Carmovirus	Carnation mottle virus
		Necrovirus	Tobacco necrosis virus A
		Dianthovirus	Carnation ringspot virus
		Machlomovirus	Maize chlorotic mottle virus
		Avenavirus	Oat chlorotic stunt virus
		Aureusvirus	Pothos latent virus
		Panicovirus	Panicum mosaic virus
Nidovirales	Coronaviridae	Coronavirus	Infectious bronchitis virus
		Torovirus	Equine torovirus
	Arteriviridae	Arterivirus	Equine arteritis virus
	Roniviridae	Okavirus	Gill-associated virus
	Togaviridae	Alphavirus	Sindbis virus
		Rubivirus	Rubella virus
	Flaviviridae	Flavivirus	Yellow fever virus
		Pestivirus	Bovine viral diarrhea virus
		Hepacivirus	Hepatitis C virus
	Bromoviridae	Alfamovirus	Alfalfa mosaic virus

Order	Family	Genus	Type Species Example
		Ilarvirus	Tobacco streak virus
		Bromovirus	Brome mosaic virus
		Cucumovirus	Cucumber mosaic virus
		Oleavirus	Olive latent virus 2
	Closteroviridae	Closterovirus	Beet yellows virus
		Crinivirus	Lettuce infectious yellows virus
		Ampelovirus	Grapevine leafroll-associated virus 3
	Barnaviridae	Barnavirus	Mushroom bacilliform virus
	Luteoviridae	Luteovirus	Barley yellow dwarf virus-PAV
		Polerovirus	Potato leafroll virus
		Enamovirus	Pea enation mosaic virus-1
		Tobamovirus	Tobacco mosaic virus
		Tobravirus	Tobacco rattle virus
		Hordeivirus	Barley stripe mosaic virus
		Furovirus	Soil-borne wheat mosaic virus
		Pomovirus	Potato mop-top virus
		Pecluvirus	Peanut clump virus
		Benyvirus	Beet necrotic yellow vein virus
		Idaeovirus	Raspberry bushy dwarf virus
		Capillovirus	Apple stem grooving virus
		Trichovirus	Apple chlorotic leaf spot virus
		Sobemovirus	Southern bean mosaic virus
		Umbravirus	Carrot mottle virus
	Tymoviridae	Tymovirus	Turnip yellow mosaic virus
		Marafivirus	Maize rayado fino virus
		Maculavirus	Grapevine fleck virus
		Carlavirus	Carnation latent virus
		Potexvirus	Potato virus X

Order	**Family**	**Genus**	**Type Species Example**
		Allexivirus	Shallot virus X
		Foveavirus	Apple stem pitting virus
		Vitivirus	Grap vine virus A
		Ourmiavirus	Ourmia melon virus

[000139] **Table 8 – Naked RNA Viruses**

Order	Family	Genus	Type Species Example
	Narnaviridae	Narnavirus	Saccharomyces cerevisiae 20SRNA narnavirus
		Mitovirus	Cryphonectria parasitica mitovirus-1 NB631

[000140] **Table 9 - Viroids**

Order	Family	Genus	Type Species Example
	Pospiviroidae	Pospiviroid	Potato spindle tuber viroid
		Hostuviroid	Hop stunt viroid
		Cocadviroid	Coconut cadang-cadang viroid
		Apscaviroid	Apple scar skin viroid
		Coleviroid	Coleus blumei viroid 1
	Avsunviroidae	Avsunviroid	Avocado sunblotch viroid
		Pelamoviroid	Peach latent mosaic viroid

[000141] **Table 10 - Subviral Agents**

Agent	Group	Type	Subgroup/Species
Satellites	Satellite Viruses	Single-Stranded RNA Satellite Viruses	Chronic bee-paralysis satellite virus
			Tobacco necrosis satellite virus
	Satellite Nucleic Acids	Single-Stranded Satellite DNAs	Tomato leaf curl virus satellite DNA
		Double-Stranded Satellite RNAs	satellite of Saccaromyces cerevisiae M virus
		Single-Stranded Satellite RNAs	Large Satellite RNAs

[000142] **Table 11A – RNA Viruses in Disease**

Family (Viridae)	Virus Subfamily or Genus	Host: Humans, Monkeys	Host: Other Animals
Picorna	Entero	Enteritis, occasionally CNS (polio)	Enteritis
	Cardio	---	Encephalomyocarditis
	Rhino	Common Cold (many serotypes)	Respiratory
	Aphtho	---	Foot and Mouth Disease
Corona	---	Respiratory and enteric	Many different diseases in different animals
Toga	Alpha	Rare encephalitis	Equine, etc. encephalitis
	Flavi	Yellow fever, encephalitis	Equine, etc. encephalitis
	Rubi	Skin rash, German Measles (rubella)	---
	Pesti	Occasionally congenital diseases	Mucosal disease (cattle)
Retro	Type C	T-cell leukemia (HTLV-1); sarcoma (monkey)	Avian, murine, and other animal leukemias and sarcomas
	Type B	---	Murine mammary tumors
	Type D	Immune deficiency (monkey)	---
	Lenti	AIDS, encephalopathy, Immune deficiency (monkey)	Immune deficiency (cats), maedi visna (sheep), encephalopathy, arthritis (goats)
Rhabdo	Vesiculo	---	Stomatitis (cattle, swine)
	Lyssa	Rabies	Rabies
Filo	---	Hemorrhagic fever (Marburg, Ebola)	---
Arena	---	Hemorrhagic fever (Lassa)	Lymphocytic choriomeningitis (mice)
Bunya	Bunyamwera	Encephalitis (Calif. Enceph.)	Many diseases
Paramyxo	Paramyxo	Childhood respiratory, croup (parainfluenza), salivary gland (Mumps)	Newcastle disease (birds)
	Morbilli	Skin rash (measles)	Rinderpest (cattle), distemper (dogs)
	Pneumo	Childhood lower respiratory, Pneumonia	

Family (Viridae)	**Virus Subfamily or Genus**	**Host: Humans, Monkeys**	**Host: Other Animals**
		(respiratory syncitial)	
Orthomyxo	Type A	Influenza (flu)	Respiratory
	Type B	Influenza	---
Reo	Orthoreo	---	---
	Orbi	Diarrhea	Diarrhea (blue-tongue of sheep)
	Rota	Children's diarrhea	Enteric
	Cytoplasmic polyhedrosis	---	Lethal insect infection

[000143] **Table 11B – DNA Viruses in Disease**

Family (Viridae)	**Virus Subfamily or Genus**	**Host: Humans, Monkeys**	**Host: Other Animals**
Parvo	---	Aplastic anemia (humans), Fifth disease (B19)	Enteritis (dogs, cats), Encephalopathy (rats), Prenatal infections
Hepadna	---	Hepatitis	Same (woodchucks, squirrels, ducks)
Papova	Polyoma	Malignant tumors under certain specific conditions; encephalopathy	Same
	Papilloma	Warts, carcinomas	Warts, at times malignant (Shope papilloma)
Adeno	(Many serotypes)	Acute respiratory diseases, conjunctivitis	Same (occasionally oncogenic)
Herpes	Alphaherpes	Skin rash: chickenpox, varicella Cold sores, shingles (herpes simplex 1) Venereal, congential (herpes simplex 2)	--- Bovine mammalitis, etc.
	Betaherpes	Congenital malformations (cytomegalo)	Respiratory and congenital diseases
	Gammaherpes	Infectious mononucleosis, etc. (Epstein-Barr)	Marek's disease (chickens)
Baculo	(nuclear polyhedrosis, granulosis)	---	Lethal insect infections
Pox	(Many Genera)	Smallpox; Yaba (monkey)	Pox, myxomatosis
Unclassified	---	Non-A, non-B hepatitis, liver cancer	---